Chapter 18

Glycosyltransferase Activity Assay Using Colorimetric Methods

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Abstract

The glycosyltransferases (GTs) are an important subclass of enzymes that catalyze the biosynthesis of glycosidic bonds in oligosaccharides, polysaccharides and glycoconjugates by transferring a sugar residue from a donor substrate to an acceptor substrate. The membrane-associated GTs play a vital role in the biosynthesis of bacterial cell-wall polysaccharides. Characterization and quantification of GT activities is important for studies of biosynthesis of polysaccharides, drug target development, and production of bacterial products. In this chapter, colorimetric assays for the measurement of GT activities will be presented. Assays for GTs acting on monosaccharide-derivatives are based on the cleavage of unreacted glycosyl-*p*-nitrophenol acceptors followed by detection of *p*-nitrophenolate. GT reactions coupled with phosphatases and detection of inorganic phosphate are suitable for most GTs. These assays permit convenient quantification of GT activities and kinetics without the use of radioactive sugars.

Key words Bacterial glycosyltransferases, Substrate specificity, Polysaccharide glycosyltransferases, Colorimetric assay, Phosphate-coupled assay

1 Introduction

Microbial polysaccharides are extremely abundant and varied in structure. Monosaccharides and oligosaccharides in the form of glycoconjugates are widespread in microorganisms, and play substantial roles in cell wall structure, antigenicity, and resistance to antibiotics [1–4]. Glycosidic bonds are synthesized by a vast number of glycosyltransferases (GTs). GTs have been classified into more than 100 families in the Carbohydrate-Active Enzyme (CAZy) data base [5]. In addition, CAZy lists glycohydrolases that break down complex carbohydrates and glycoconjugates and also play important biological roles [6]. GTs are the enzymes responsible for the biosynthesis of simple and complex glycans by transferring a sugar residue from a specific phospho-activated sugar donor to an acceptor substrate [7–9]. Usually, GTs are highly specific for both, their donor and acceptor substrates. Depending

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on the GT specificity, monosaccharides, disaccharides, and oligosaccharides, as well as proteins, lipids, DNAs, and numerous small molecules and their derivatives are acceptor substrates [9]. Consequently, GTs play a crucial role in biosynthesis pathways of oligosaccharides, polysaccharides and glycosylated proteins and lipids.

Notwithstanding their abundance and important functions, assaying GTs remains challenging for glycoscientists. The scientific hurdle has been the lack of availability of defined acceptor substrates and complex donor substrates required for bacterial GTs. Synthetic compounds help to overcome this hurdle (*see* Chapters 12, 13, 15, 17, 19). GTs can be evaluated by the transfer of a radiolabeled sugar from a donor to an acceptor substrate [10, 11] followed by the separation and quantification of radiolabeled reaction products using chromatography methods such as column or thin layer chromatography. Several nonradioactive methods have also been reported for assaying GTs [12–15].

In this chapter, we describe nonradioactive colorimetric methods for assaying GTs. The first colorimetric assay method is suitable for GTs that require only one sugar in the acceptor substrate. As an example, the glycosyl acceptor for certain Gal-transferases is GlcNAcβ-4-nitrophenol (GlcNAcβ-*p*NP), and for certain GlcNAc-transferases is Galβ-*p*NP. While the GT product is resistant to hydrolysis by exoglycosidases, the monosaccharide acceptor is hydrolyzed by a specific exoglycosidase to release *p*NP which is quantified by measuring its visible absorption. The enzymatic reactions for the GT reaction and release of *p*NP from the acceptor substrate are shown in Fig. 1. This GT activity assay is comparatively easy, simple, sensitive, and quantitative. However, the method is limited to enzymes that can utilize glycosyl-*p*NP as acceptor substrate such as GlcNAc-transferase LgtA and Gal-transferases LgtB and LgtC from *Neisseria meningitides* [14]. Other bacterial GTs may require more complex oligosaccharide acceptor substrates [10, 11].

A method that is broadly applicable to most GTs is a Phosphatase-coupled assay [15]. One of the GT reaction products is a nucleotide (e.g., UDP, GDP, or CMP). The terminal phosphate of this reaction product can be acted on by a phosphatase, thus releasing inorganic phosphate that can be quantified with a color reagent. The enzymatic reactions for releasing free phosphate are shown in Fig. 2. This method is unrelated to the structure of the acceptor substrate and thus is applicable to most GTs, including LgtA, LgtB and LgtC. However, it is recommended that purified enzyme preparations are used that do not have contaminating phosphatases.

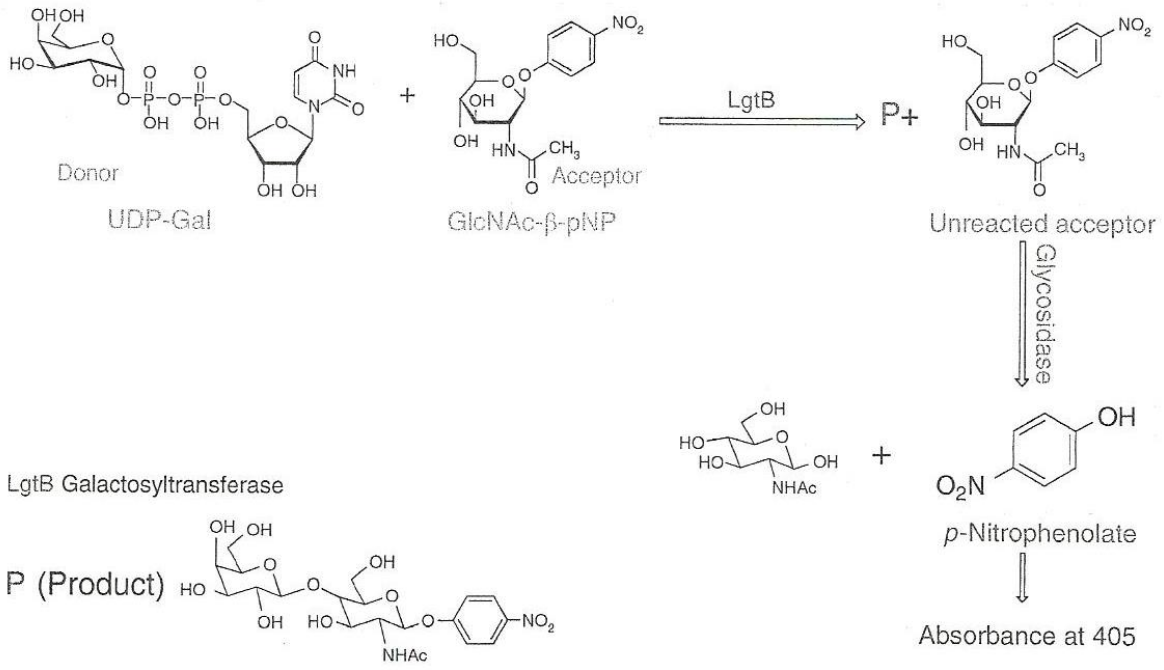

Fig. 1 Colorimetric screening method for glycosyltransferase activity. After transfer of the Gal residue to GlcNAcβ-*p*NP by Gal-transferase LgtB the unreacted acceptor is cleaved by exo-β-*N*-acetyl-hexosaminidase, releasing *p*-Nitrophenolate which is detected by measuring the change of visible absorption at 405 nm

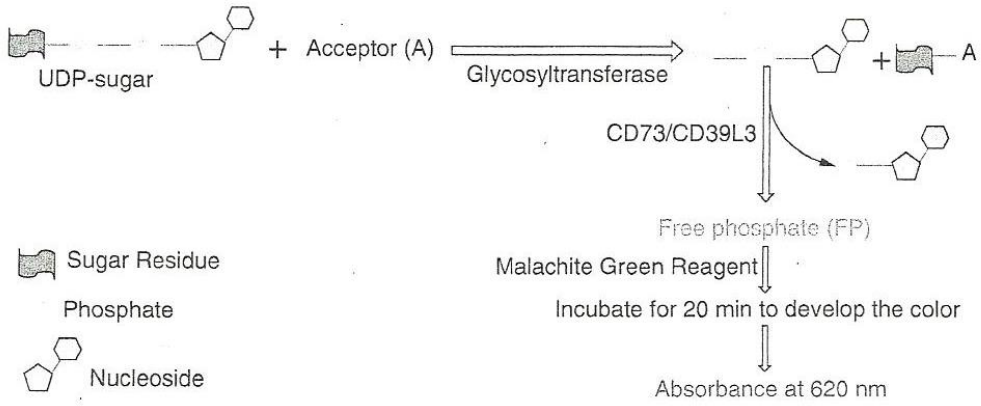

Fig. 2 Phosphatase-coupled screening for glycosyltransferase activity. This approach can be applied to any glycosyltransferase reaction with a removable phosphate-containing leaving group. In the glycosyltransferase reaction mixture, a phosphonucleotide reaction product is acted upon by a coupling phosphatase CD73 or CD39L3. The inorganic phosphate is detected using phosphate detection reagents. Quantification is done from a standard curve with various concentrations of phosphate

2 Materials

All the reagents and microbial cultures (if needed) are prepared under aseptic conditions (*see* **Note 1**). The reagents and buffers are prepared by using deionized, doubly distilled or milli-Q water at room temperature and the reagents are stored at room temperature (unless indicated otherwise). Always use analytical or reagent grade chemicals.

2.1 Colorimetric Assay for Gal-transferases (See Note 2)

1. 0.5 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) buffer: Dissolve 11.92 g Hepes in water, adjust to pH 7.2 and add water to make up to 100 mL.
2. 0.1 M 4-nitrophenyl-*N*-acetyl-β-D-glucosaminide (GlcNAc-β-*p*NP) solution (Sigma).
3. 10 mM uridine diphosphate galactose (Sigma).
4. 0.1 M $MnCl_2$.
5. As an example, recombinant Gal-transferase LgtB from *Neisseria meningitides* is expressed in *E. coli* BL21 and purified [14]. It is then dialyzed against 20 mM phosphate-buffered saline (PBS) pH 7.5 (*see* **Note 3**). The enzyme is stored in 50% glycerol in 10 mM PBS at −20 °C.
6. Bovine serum albumin (BSA).
7. 0.5 M Na_2CO_3.
8. Exo-*N*-acetylglucosaminidase (Sigma).
9. Microplate reader.

2.2 Phosphatase-Coupled Glycosyltransferase Assay

1. Reaction buffer: 0.1 M Tris–HCl, pH 7.5.
2. 0.15 M NaCl.
3. 0.1 M $MgCl_2$.
4. 0.1 M $MnCl_2$.
5. 10 mM UDP-Gal.
6. Acceptor substrate: 0.1 M GlcNAcβ–benzyl (Sigma).
7. Purified Gal-transferase LgtB [14] (*see* **Note 4**).
8. Coupling phosphatase (ecto-5′-nucleotidase CD73) solution: Dissolve 1.0 mg CD73 (Sigma) in 1.0 mL reaction mixture.
9. Malachite Green Phosphate Detection Kit (R&D Systems, Minneapolis, USA).
10. Microplate reader.

3 Methods

3.1 Colorimetric Assay for Gal-transferases

1. Prepare a reaction mixture on 96 well microplates in a total volume of 100 μL by adding 10 μL 0.5 M Hepes buffer (pH 7.2), 1 μL 0.1 M GlcNAc-β-*p*NP, 5 μL 0.1 M $MnCl_2$, 4 μL 10 mM UDP-Gal, 10 μL purified glycosyltransferase (in 10 mM PBS/50% glycerol), 10 μL BSA (0.1 mg/mL) (*see* **Note 5**) and 60 μL water. All assays are done in triplicate and the average values will be determined. Control assays lack the Gal-transferase but have 10 mM PBS/50% glycerol instead. Another set of control assays lack UDP-Gal.
2. Mix the solution gently.
3. Incubate at 37 °C for 30 min.
4. Add an excess (1 unit) of exo-N-acetylhexosaminidase to cleave unreacted GlcNAcβ-*p*NP and quench the reaction.
5. Incubate at 37 °C for 30 min.
6. Adjust the final pH to 9.6 by adding 0.5 M Na_2CO_3.
7. Monitor the *p*-nitrophenolate by measuring absorbance at 405 (A_{405}). The absorbance of the *p*-nitrophenolate is directly correlated to the amount of unreacted GlcNAcβ-*p*NP in the GT reaction mixture and to the GT activity. For comparison, a standard curve is made using different concentrations of *p*NP under the assay conditions but lacking enzymes and donor substrates. Subtract values from control assays and calculate activity (μmol × min^{-1} × mg $protein^{-1}$).

3.2 Phosphatase-Coupled Glycosyltransferase Assay

1. In a 96-well plate, prepare a reaction mixture in a total volume of 50 μL: Add 12 μL 0.1 M Tris–HCl, pH 7.5, 10 μL 150 mM NaCl, 2 μL 0.1 M $MgCl_2$, 2 μL 0.1 M $MnCl_2$, 2 μL 0.1 M GlcNAcβ-benzyl acceptor (*see* **Note 6**), 10 μL purified LgtB in 10 mM PBS/50% glycerol. Vortex the mixture gently. Initiate reaction with 5 μL 10 mM UDP-Gal and 0.1 μg coupling phosphatase CD73. Add water to make up to 50 μL (*see* **Note 7**).
2. Incubate for 30 min at 37 °C.
3. Quench the reaction by adding 30 μL of Malachite Reagent A and 100 μL of water to each well.
4. Add 30 μL of Malachite Green Reagent B to develop color, followed by gentle mixing.
5. Prepare control assays under the same conditions but without Gal-transferase.
6. Perform all measurements in triplicate.
7. Incubate at room temperature for 20 min.

8. Read the absorbance at 620 nm with a plate reader.
9. Average the values and subtract values from control assays.
10. Perform a phosphate standard curve to determine the conversion factor between the absorbance and the inorganic phosphate contents. The concentrations of phosphate groups in the assay mixtures are determined by the standard curve using different concentrations of phosphate. The GT activity is then determined (μmol phosphate produced x min^{-1} x mg GT $protein^{-1}$).

4 Notes

1. Aseptic technique is a set of principles and practices used for bacterial culture preparation and transferring culture to reduce undesirable microbes.
2. This protocol is presented to assay a Gal-transferase that acts on GlcNAcβ-*p*NP as acceptor substrate but other Gal-transferases may also be assayed with this method. The GT should be purified but bacterial homogenates may also have significant GT activity. Other glycosyl-*p*NP acceptors could be used (e.g., Galβ-*p*NP together with UDP-GlcNAc donor substrate to assay GlcNAc-transferase LgtA from *Neisseria meningitides*) [14]. In this case, an exo-β-galactosidase is used to liberate *p*NP.
3. Other GTs can be expressed in BL21 cells, but the purification procedure may be adjusted according to the affinity tag they carry. It is important that they can utilize Glycosyl-*p*NP as an acceptor substrate. The assays are not suitable for GTs that require a glycosyl-diphosphate-lipid or complex oligosaccharide as acceptors.
4. To obtain kinetic parameters for UDP-Gal the concentration should be varied from 0.005 to 1 mM and a fixed saturating concentration of GlcNAcβ-*p*NP (2 mM).
5. BSA is added to stabilize highly purified GTs.
6. Many other acceptors are appropriate, depending on the specificity of the GT, such as GlcNAcβ-*p*NP or more complex oligosaccharide derivatives.
7. This method is a general method for GTs and a variety of donor substrates. Thus, GT enzymes and their donor and acceptor substrates could be changed.

Acknowledgments

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