Analytical Biochemistry 447 (2014) 146–155

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Development of a highly sensitive, high-throughput assay for glycosyltransferases using enzyme-coupled fluorescence detection

Kazuo Kumagai [a,b,*], Hirotatsu Kojima [a], Takayoshi Okabe [a], Tetsuo Nagano [a]

[a] Open Innovation Center for Drug Discovery, The University of Tokyo, Tokyo 113-0033, Japan
[b] Genomic Science Laboratories, Dainippon Sumitomo Pharma Co. Ltd., Osaka 554-0022, Japan

ARTICLE INFO

Article history:
Received 10 August 2013
Received in revised form 20 November 2013
Accepted 22 November 2013
Available online 1 December 2013

Keywords:
Glycosyltransferase
Nucleotide determination
Enzyme-coupled assay
Fluorescence assay
High-throughput screening

ABSTRACT

Glycosyltransferases catalyze transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds. Identification of selective modulators of glycosyltransferases is important both to provide new tools for investigating pathophysiological roles of glycosylation reactions in cells and tissues, and as new leads in drug discovery. Here we describe a universal enzyme-coupled fluorescence assay for glycosyltransferases, based on quantification of nucleotides produced in the glycosyl transfer reaction. GDP, UDP, and CMP are phosphorylated with nucleotide kinase in the presence of excess ATP, generating ADP. Via coupled enzyme reactions involving ADP-hexokinase, glucose-6-phosphate dehydrogenase, and diaphorase, the ADP is utilized for conversion of resazurin to resorufin, which is determined by fluorescence measurement. The method was validated by comparison with an HPLC method, and employed to screen the LOPAC1280 library for inhibitors in a 384-well plate format. The assay performed well, with a Z′-factor of 0.80. We identified 12 hits for human galactosyltransferase B4GALT1 after elimination of false positives that inhibited the enzyme-coupled assay system. The assay components are all commercially available and the reagent cost is only 2 to 10 US cents per well. This method is suitable for low-cost, high-throughput assay of various glycosyltransferases and screening of glycosyltransferase modulators.

Glycosyltransferases are ubiquitous enzymes that are involved in the biosynthesis of disaccharides, oligosaccharides, and polysaccharides. They catalyze the transfer of a sugar moiety from donor molecules to glycosyl acceptor molecules, which range from proteins, lipids, sugars, and nucleic acids to small molecules [1]. Defects in glycosyltransferase activities are associated with various human diseases, including cancer, inflammation, neurological disorders, and congenital disorders of glycosylation [2,3]. Glycosyltransferases also influence the distribution and clearance of various biological molecules by regulating their glycosylation [4,5]. Thus, small-molecular modulators of glycosyltransferases are expected to be useful research tools and may also serve as leads for drug discovery. However, few potent and selective inhibitors of glycosyltransferases are known [6], largely due to the lack of suitable high-throughput screening (HTS)[1] assay methods to screen large chemical libraries.

Currently, the most widely used assay methods for glycosyltransferases are based on the detection of glycosylated products by using chromatographic, radiochemical, spectrophotometric, or immunological techniques [7]. However, most of these methods are low throughput, expensive, and labor-intensive. Compared with these methods, an alternative approach based on determination of nucleoside di(mono)phosphate generated from the sugar donor substrate would be both simpler and generally applicable to all glycosyltransferases. Most glycosyltransferases in mammals utilize uridine diphosphate (UDP)-sugars (e.g., galactosyltransferases), guanosine diphosphate (GDP)-sugars (e.g., fucosyltransferases), or cytidine monophosphate (CMP)-sugars (e.g., sialyltransferases) as sugar donor substrates [8]. Hence, the nucleotides UDP, GDP, and CMP liberated from these glycosyl donors are common products of glycosyltransferase reactions. Several methods other than chromatographic methods have been developed to detect these nucleotides for glycosyltransferase activity assays. Fluorescence polarization-based assays have been developed for the quantification of UDP, GDP, and CMP, and are available as commercial assay kits, but they are expensive for use in HTS [7]. Wu et al. determined inorganic phosphate released from these nucleotides by nucleotide phosphatase reactions by means of colorimetry with a malachite-based reagent [9]. This method is less costly and applicable to a wide range of glycosyltransferases, but

* Corresponding author. Address: Open Innovation Center for Drug Discovery, The University of Tokyo, Pharmaceutical Science Main Building, Tokyo 113-0033, Japan. Fax: +81 3 5841 1959.
E-mail address: kazuo-kumagai@mol.f.u-tokyo.ac.jp (K. Kumagai).

[1] *Abbreviations used:* HTS, high-throughput screening; B4GALT1, β-1,4-galactosyltransferase 1; FUT7, fucosyltransferase 7; ST6GAL1, β-galactoside α-2,6-sialyltransferase 1; NDP, nucleoside 5′-diphosphate; NDPK, nucleoside diphosphate kinase; CMPK1, CMP kinase 1; CMP-NeuAc, CMP-sialic acid; G6PDH, glucose-6-phosphate dehydrogenase; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide.

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.11.025

is not sensitive. Zhang et al. reported an enzyme-coupled fluorescence assay for *N*-acetylglucosaminyltransferase activity in which UDP was determined fluorometrically after coupling with pyruvate kinase reaction: detection was done by a commercial pyruvate assay kit [10]. UDP, GDP, and CMP can also be determined spectrophotometrically or fluorometrically by enzyme-coupled NADH detection [11–13], but the fluorescence intensity of NADH is relatively low.

Here we report a highly sensitive, easy-to-operate, universal assay based on enzyme-coupled fluorescence detection of UDP, GDP, and CMP. This method is applicable in principle to any glycosyltransferase that utilizes UDP-, GDP-, or CMP-sugars as substrates. It employs nucleotide kinase reaction to generate ADP from the three nucleotides, followed by a cascade of coupled enzyme reactions that utilize the ADP for conversion of resazurin to highly fluorescent resorufin, which is determined by fluorescence measurement. The optimized method requires a volume of only a few microliters per well for glycosyltransferase assay in a 384-well plate format, and provides a high signal-to-background ratio. The method was validated by comparison with HPLC assay, and was applied to screen the LOPAC1280 library for inhibitors of human galactosyltransferase B4GALT1. We believe that this assay system provides a simple, cost-effective tool that will be applicable to many glycosyltransferases.

Materials and methods

Materials

Recombinant human β-1,4-galactosyltransferase 1 (B4GALT1), fucosyltransferase 7 (FUT7), and β-galactoside α-2,6-sialyltransferase 1 (ST6GAL1) were purchased from R&D Systems (Minneapolis, MN, USA). Nucleoside diphosphate kinase (NDPK, 1.9 kU/mg protein) from baker's yeast, fetuin from fetal bovine serum, *N*-acetyl-D-lactosamine, and the LOPAC1280 compound library were obtained from Sigma–Aldrich (St. Louis, MO, USA). Recombinant human cytidine monophosphate kinase 1 (CMPK1) was from Prospec (Ness Ziona, Israel). ADP-hexokinase (52.6 U/mg solid) from *Thermococcus litoralis* was from Asahi Kasei Pharma (Tokyo, Japan). Diaphorase-I (1.8 kU/mg protein) from *Bacillus stearothermophilus* was from Unitika (Osaka, Japan). Recombinant glucose-6-phosphate dehydrogenase (G6PDH, 754 U/mg protein) from *Leuconostoc* sp. and $NADP^+$ were from Oriental Yeast (Tokyo, Japan). GDP-fucose (GDP-Fuc), UDP-galactose (UDP-Gal), and CMP-sialic acid (CMP-NeuAc) were from Yamasa (Choshi, Japan), MP Biomedicals (Solon, OH, USA), and Calbiochem (San Diego, CA, USA), respectively. ATP, GDP, UDP, dithiothreitol (DTT), *N*-acetylglucosamine (GlcNAc), *N*-ethylmaleimide (NEM), bovine serum albumin (BSA), resazurin, gallic acid, and other chemicals were from Wako Pure Chemical Industries (Osaka, Japan). Nonbinding, small-volume 384-well black microtiter plates were from Greiner Bio-One (Frickenhausen, Germany).

Determination of ADP by enzyme-coupled fluorescence assay

ADP in 7.5 μl of 50 mM Tris-HCl (pH 7.5) was mixed with 7.5 μl aliquots of 2X ADP detection reagent (2 mM glucose, 200 μM $NADP^+$, 100 μM resazurin, 2 U/ml ADP-hexokinase, 2 U/ml G6PDH, and 2 U/ml diaphorase I in buffer A (100 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5)) in 384-well plates and incubated at room temperature for 60 min. Fluorescence intensity due to the formation of resorufin was measured with a microplate reader, PHERAstar (BMG Labtech, Offenburg, Germany), with excitation at 540 nm and emission at 590 nm.

Enzyme-coupled fluorescence assay of GDP-fucosyltransferase and UDP-galactosyltransferase

The reactions of fucosyltransferase FUT7 and galactosyltransferase B4GALT1 were performed in 5 μl of buffer B (50 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, pH 7.5) in 384-well plates at 37 °C for 60 min. FUT7 was reacted with 3 mg/ml fetuin and 50 μM GDP-Fuc. B4GALT1 was reacted with 5 mM GlcNAc and 50 μM UDP-Gal. To detect GDP and UDP produced by these reactions, 5 μl aliquots of 200 μM ATP and 2 U/ml NDPK in buffer A were added and the mixtures were incubated at room temperature for 60 min. Then 10 μl aliquots of 2X ADP detection reagent were added and incubation was continued at room temperature for 60 min. The fluorescence intensity (ex 540/em 590) was measured as described above.

Enzyme-coupled fluorescence assay of CMP-sialyltransferase

Sialyltransferase ST6GAL1 reaction was carried out by adding the enzyme to 5 μl of buffer C (25 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 150 mM NaCl, 0.1% (w/v) Triton X-100, pH 7.5) supplemented with 250 μM *N*-acetyl-D-lactosamine and 50 μM CMP-NeuAc in 384-well plates at 37 °C for 90 min. To detect CMP produced by the reaction, 5 μl aliquots of 200 μM ATP, 3 μg/ml CMPK1, and 4 mM DTT in buffer D (175 mM Tris-HCl, 20 mM $MgCl_2$, 150 mM KCl, 0.01% (w/v) BSA, 0.1% (w/v) Triton X-100, pH 9.0) were added. Incubation was continued at 37 °C for 60 min, and then 10 μl aliquots of 2X ADP detection reagent supplemented with 20 mM NEM were added. After further incubation at room temperature for 60 min, the fluorescence intensity (ex 540/em 590) was measured.

HPLC analysis

HPLC analysis for nucleotides and nucleotide-sugars was performed using a YMC-Triart C18 column (150 × 4.6 mm, S-5 μm, YMC, Kyoto, Japan) with 50 mM KH_2PO_4–K_2HPO_4 (100:0 (v/v) for CDP, CMP, and CMP-NeuAc; 67:33 (v/v) for ADP, ATP, GDP, GTP, GDP-Fuc, UDP, UTP, and UDP-Gal) as an eluent at a flow rate of 1 ml/min with UV detection at 260 nm.

Assay validation

Assay of inhibitory activity toward FUT7 was performed by both the enzyme-coupled fluorescence method and the HPLC method for validation of the new method. FUT7 (1 μg/ml) was incubated with 3 mg/ml fetuin and 50 μM GDP-Fuc in the presence or absence of the test compound gallic acid in 20 μl of buffer B at 37 °C for 60 min. The reaction was stopped by chilling the tubes on ice, and samples (10 μl) were analyzed by HPLC to determine liberated GDP. Remaining samples (5 μl) were transferred to wells of a 384-well plate. Aliquots of 5 μl of 200 μM ATP and 2 U/ml NDPK in buffer A supplemented with 0.01% (w/v) BSA were added and the plate was incubated at room temperature for 60 min. Then 10 μl of 2X ADP detection reagent supplemented with 0.01% (w/v) BSA was added to each well and incubation was continued at room temperature for 60 min, followed by fluorescence measurement. The percentage inhibition was calculated relative to controls treated with DMSO alone. In the control assay to determine the effect of the test compound on the enzyme-coupled assay reactions, 5 μl aliquots of 20 μM GDP in buffer B in the presence or absence of test compound were pipetted into wells of a 384-well plate instead of the FUT7 reaction mixtures, and enzyme-coupled fluorescence detection of GDP was performed as described above.