Journal of Biotechnology 257 (2017) 162–170

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Development of a colourimetric assay for glycosynthases

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ARTICLE INFO

Article history:
Received 1 October 2016
Received in revised form 7 February 2017
Accepted 7 February 2017
Available online 11 February 2017

Keywords:
Glycosynthase
Chemosensor
Fluoride detection
Enzyme characterisation
Screening

ABSTRACT

The synthesis of glycosidic structures by catalysis via glycosynthases has gained much interest due to the potential high product yields and specificity of the enzymes. Nevertheless, the characterisation and implementation of new glycosynthases is greatly hampered by the lack of high-throughput methods for reaction analysis and screening of potential glycosynthase variants. Fluoride detection, via silyl ether chemosensors, has recently shown high potential for the identification of glycosynthase mutants in a high-throughput manner, though limited by the low maximal detection concentration. In the present paper, we describe a new version of a glycosynthase activity assay using a silyl ether of *p*-nitrophenol, allowing fast reliable detection of fluoride even at concentrations of 4 mM and higher. This improvement of detection allows not only screening and identification but also kinetic characterisation of glycosynthases and synthetic reactions in a fast microtiter plate format. The applicability of the assay was successfully demonstrated by the biochemical characterisation of the mesophilic β-glucosynthase of Abg-E358S (*Rhizobium radiobacter*) and psychrotolerant β-glucosynthase BglU-E377A (*Micrococcus antarcticus*). The limitation of hyperthermophilic glycosidases as potential glycosynthases, when using glycosyl fluoride donors, was also illustrated by the example of the putative β-galactosidase GalPf from *Pyrococcus furiosus*.

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1. Introduction

Naturally occurring glycosidic compounds are often of high interest in pharmaceutical, food and detergent industry. The synthesis and analysis of these structures can support: a) the understanding of cellular recognition structures and antigenic compounds, b) the development of new bioactive compounds (*e.g.* anti-cancerogenous compounds etc.), c) diversification of food and aroma, and d) the production of higher performing washing detergents. The use of enzymes such as glycosyl transferases (E.C. 2.4.1.n) and especially glycosidases (E.C. 3.2.1.n) for the synthesis of glycosides has increased much in past years (Danby and Withers, 2016; Trincone, 2015). The mechanisms of these enzymes in hydrolysis and synthesis have been extensively researched, allowing many advances in synthesis by both, complex genetic engineering and structural examination (Shaikh and Withers, 2008; Talens-Perales et al., 2016). Glycosyl transferases have been utilised successfully in synthetic reactions; though, the major drawback of this method being the high cost of hardly available nucleotide donors and the difficult heterologous production of these enzymes (Henze et al., 2015; McArthur and Chen, 2016). Methods using natural glycosidases, such as reverse hydrolysis (De Winter et al., 2013) or transglycosylation have been also successfully used for the synthesis of various glycoside building blocks (Bissaro et al., 2015). However, the natural hydrolytic activity can lead to low yields of synthetic reactions due to hydrolysis of the received glycoside product. An alternative to these natural enzymes are glycosyn-

Abbreviations: wt, wild type; Abg, *Agrobacterium* β-glucosidase; BglU, β-glucosidase U; GalPf, galactosidase *P. furiosus*; LB, lysogeny broth; TB, terrific broth; TLC, thin layer chromatography; DMF, dimethyl formamide; TBS, *tert*-butyldimethylsilyl; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; pNP, *p*-nitrophenol; sat., saturated; TIPS, triisopropylsilyl; LOD, limit of detection; LOQ, limit of quantitation; pNPGlc, *p*-nitrophenyl α-D-glucopyranoside; α-GlcF, α-D-glucopyranosyl fluoride; GlcNAc, *N*-acetyl-glucosamine; KP_i, KH_2PO_4/K_2HPO_4; U, units = μmol/min.

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http://dx.doi.org/10.1016/j.jbiotec.2017.02.005
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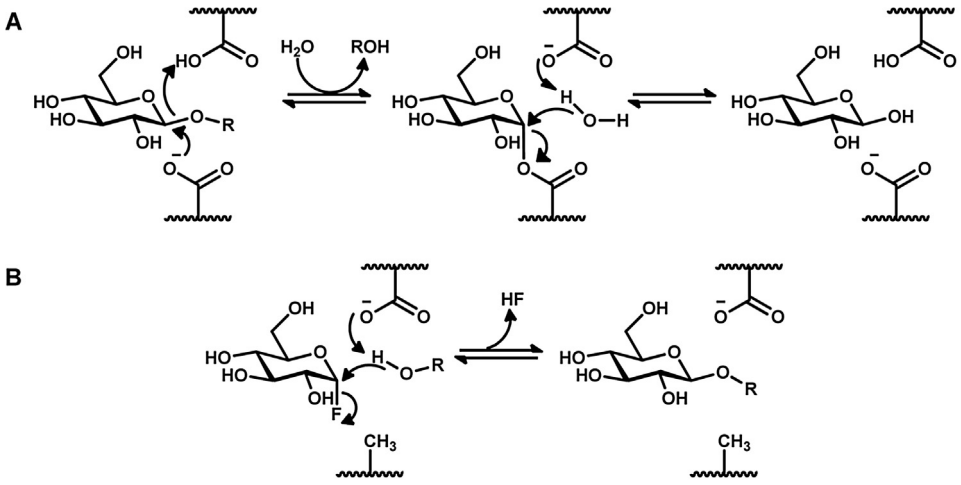

Scheme 1. Comparison of the mechanism of a natural glycosidase (A) and unnatural glycosynthase (B). The nucleophilic residue is replaced by a non-nucleophilic residue (here: alanine) in order to eliminate hydrolytic activity.

thases, which are genetically engineered glycosidases mutated in the active site of the enzyme (Mackenzie et al., 1998). These unnatural enzymes lack the catalytic active nucleophilic residue in the active site. Consequently, these enzymes are void of hydrolytic activity. Nevertheless, the intact structure of the enzyme allows the transfer of activated glycosyl donors [such as glycosyl fluorides or azides (Cobucci-Ponzano et al., 2011)] onto acceptors with complete anomeric control (Scheme 1).

The use of these enzymes in synthetic reactions has led to higher yields of various glycosidic structures, than with glycosidases, as the synthesized product cannot be hydrolysed by the enzyme (Ben-David et al., 2007; Goddard-Borger et al., 2016; Henze et al., 2015). Nevertheless, identification and characterisation of potential glycosynthases in mutant libraries or for synthetic reactions proves a laborious task due to the lack of high-throughput assay methods for these enzymes. Standard analytical methodology is complex and lengthy reverse HPLC analyses of the enzymatic reaction with respect to product formation (Codera et al., 2016; Pozzo et al., 2014). Alternatively, measurement of the liberated proton by means of pH-indicators has also been attempted to determine glycosynthase activity (Ben-David et al., 2008). Yet this type of assay is strongly dependent on the pK_a value of the indicator limiting the type of buffer applicable for the particular indicator. Also, when using for example cell lysates for synthesis, many disturbing factors can cause noisy measurements. Recently enzyme-coupled- and ELISA-based assays have been developed, though with narrow applicability due to the high specificity towards the examined enzyme (Danby and Withers, 2016; Hancock et al., 2008). The most commonly used method is the ion-selective electrode which detects the released fluoride by conductivity measurement (Honda et al., 2016). Despite the simplicity of the method, the bulkiness of the electrode necessitates a large reaction volume and parallel measurements are hardly possible. On the other hand, fluoride chemosensors, which are found in many variations throughout literature, give a possibility of developing a high-throughput assay, which are highly selective and show little disturbing factors (Sokkalingam and Lee, 2011; Zhou et al., 2014). These chemosensors, mostly large organic compounds, are most commonly used for determination of fluoride concentrations in water samples or visualisation of fluoride in cellular systems. The weak solubility of these compounds in aqueous media often limits their use for biocatalysis due to the requirement of organic (co-)solvents. A new type of discontinuous glycosynthase assay utilising a silyl ether of methylumbelliferone was developed by Andrés et al. (2014). The chemosensor (TBS-protected methylumbelliferone) was added to a prior incubated reaction mixture containing a cell-free lysate (of a mutagenesis library) and the glycosyl fluoride donor. The release of methylumbelliferone was quantified via fluorescence measurements in order to identify potential glycosynthases. Further characterisation of the identified mutants was then carried out by HPLC analysis due to the detection limitation of the assay. The work presented in this paper, describes a modified version of this type of chemosensor assay in which the fluoride quantification is carried out using triisopropyl-(4-nitrophenoxy)-silane (**2**) in aqueous acetonitrile. A set of three glycosidases, Abg (*R. radiobacter*, mesophilic), BglU (*M. antarcticus*, psychrotolerant) and GalPf (*P. furiosus*, hyperthermophilic) were chosen and mutated to potential glycosynthases for proof of applicability of the developed assay. Additional to the determination of activity, the determination of kinetic parameters, conversion, and the screening of the acceptor scope of glycosynthases is demonstrated by use of the developed assay.

2. Material and methods

2.1. Bacterial strains, chemicals and media

E. coli DH5α (Meselson, 1968), *E. coli* BL21(DE3) (Studier and Moffatt, 1986), and *E. coli* Rosetta 2^{TM} (Moffatt and Studier, 1987) were used for replication of DNA and protein production, respectively. Chemicals for synthesis, 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose, HF/pyridine (70%, w/w), imidazole, triisopropylsilyl chloride, 4-nitrophenol, ammonia/methanol (7 M) were purchased in high purity from *Carbolution chemicals* (Saarbrücken), *Sigma-Aldrich* (Steinheim), *Alfa Aesar GmbH & Co. KG* (Karlsruhe), and *Carl Roth GmbH + Co. KG* (Karlsruhe). Solvents used in synthesis were purified before use via distillation and dried when needed by distillation over an adequate drying agent. Substrates for enzymatic assaying and biological experiments were also purchased from *Alfa Aesar GmbH & Co. KG* (Karlsruhe), *Carl Roth GmbH + Co. KG* (Karlsruhe), *Sigma-Aldrich* (Steinheim), *Fermentas GmbH* (St. Leon-Rot), and *Perla Biotechnology GmbH* (Erlangen). Cultivation of bacteria was carried out in LB-medium containing 1% (w/v) trypton, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, and protein expression in TB-medium containing 1.2% (w/v) trypton, 2.4% yeast extract, 0.4% (v/v) glycerol, 0.2% (w/v) KH_2PO_4, and 1.3% (w/v) K_2HPO_4.

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