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Chemical and Enzymatic Synthesis of Glycoconjugates 2. High Yielding Regioselective Synthesis of N-Acetyllactosamine by Use of Recombinant Thermophilic Glycosidases Library

Jun Li and Peng G. Wang*

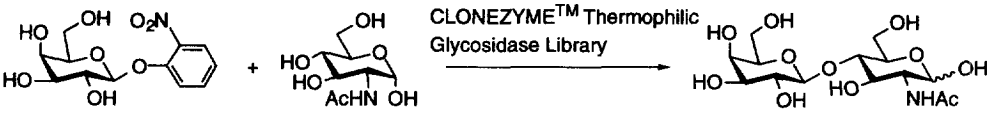
Department of Chemistry, Wayne State University, Detroit, MI 48202-3489

Abstract: β-Galactosidase activities from the recombinant thermophilic CLONEZYME™ glycosidase library were screened at 70 °C for catalysis of a transgalactosylation from *o*-nitrophenyl-β-galactopyranoside to N-acetylglucosamine. Three thermophilic glycosidases (Gly001-06, -07 and -09) were found to produce predominantly the β(1-4)-linked isomer, Gal β(1-4)GlcNAc with up to 61% yield and less than 10% of the hydrolysis side reaction product. Thus, commercial recombinant thermophilic enzyme libraries constitute a novel class of biocatalysts for preparative organic synthesis.

Enzymes evolving from thermophilic and hyperthermophilic organisms have attracted considerable attention due to their potential as biocatalysts with unprecedented properties for industrial applications. With the aid of recombinant technology and robotic screening approaches, vast untapped resources of thermophilic microorganisms can be accessed for the construction of novel enzyme libraries, from which unrivaled specificity and selectivity are expected.[1] The glycosidase CLONEZYME™ library which currently contains 10 unique thermostable glycosidases has been developed by Recombinant Biocatalysis Inc. (San Diego, CA) through cloning and automated high through-put screening systems.[2] Each enzyme displays a variety of activities ranging from galactosidase, glucosidase to fucosidase. However, potential synthetic applications of these novel enzymes have not yet been explored.

N-Acetyllactosamine, Galβ(1-4)GlcNAc, is one of the most fundamental oligosaccharide sequences on glycoproteins and glycolipids.[3] Much effort has been put into for the synthesis of such a sequence using chemical[4] and enzymatic[5] methods. Glycosyltransferase-catalyzed reaction is attractive because of its high regio- and stereoselectivity. Nevertheless, it is limited in terms of enzyme availability, cost and stability. In comparison, galactosidase-catalyzed transglycosylation reaction is more cost effective.[6] The use of β-D-galactosidase from *Bacillus circulans*[7] and the tandem use of galactose oxidase and galactosidase[8] for the synthesis of Galβ(1-4)GlcNAc derivatives resulted in high regioselectivity and yield. In the study of β-galactosidase catalyzed transglycosylation, the use of nitrophenyl galactopyranoside as a donor in the presence of an excess of N-acetylglucosamine as a glycosyl acceptor facilitates the formation of a transglycosyl product. It also reduces the possibility of further glycosylation of the desired product or donor-to-donor self glycosylation. Previous studies indicated that high yields, regioselectivity and low levels of hydrolysis from transglycosylation reactions are largely dependent on the source of the enzyme.[9, 10] In this study, β-galactosidase activities from the commercially available CLONEZYME™ glycosidase library were screened for the synthesis of N-acetyllactosamine.

The synthesis of N-acetyllactosamine (Scheme 1) was carried out at 70 °C using nine CLONEZYME glycosidases (Gly001-01 to Gly001-09) which all have β-galactosidase activities.



Scheme 1. Synthesis of N-acetyllactosamine with CLONEZYME glycosidase library

In comparison, β-galactosidases from mesophilic sources of *Aspergillus oryzae*, *Bacillus circulans* and *E. coli* were applied to the same reaction at room temperature. Results (Table 1) demonstrated that three of the nine thermophilic enzymes have superior features with yields of N-acetyllactosamine up to 61%. No other appreciable amount of regioisomers were observed from reactions using Gly001-06, 07 and 09 enzymes. It is noteworthy that the yield of the hydrolysis side reaction is much lower for the three enzymes, and even less than 10% for both Gly001-06 and 07 in aqueous media without any organic solvent.

Table 1. Enzymatic Synthesis with Recombinant Thermophilic Glycosidase CLONEZYME™ Library and Conventional Mesophilic β-Galactosidase.[a]

Enzyme	Time	Hydrolysis[b]	Yield of N-acetyllactosamine (%)[c]
Gly001-01	5 h	25%	
Gly001-02	30 min	92%	
Gly001-03	30 min	79%	
Gly001-04	30 min	74%	
Gly001-05	5 h	3%	
Gly001-06	30 min	9%	48%
	5 h	9%	46%
Gly001-07	30 min	8%	45%
	5 h	9%	43%
Gly001-08	30 min	86%	
Gly001-09	30 min	18%	61%
	5 h	23%	50%
β-galactosidase from			
E. coli	30 min	59%	
Aspergillus oryzae	30 min	57%	
Bacillus circulans	30 min	15%	25%
	5 h	71%	19%

[a] Transglycosylation experiments were conducted as follows: To a solution of N-acetyl glucosamine acceptor (0.9 mmol) and *o*-nitrophenyl β-D-galactopyranoside (0.15 mmol) in 3 mL of sodium phosphate buffer (50 mM, pH 6.0) was added glycosidase (0.2 mg) from CLONEZYME library. The reaction was incubated at 70 °C for 5 h. During the reaction, 100 μL of each sample were collected every hour and immediately placed in the freezer. After centrifugation, the supernatants were analyzed by HPLC. The product of the transgalactosylation reaction, Galβ(1-4)GlcNAc, was isolated from a preparative scale reaction using the same concentration of Gly001-09 as above and identified by ^{1}H and ^{13}C NMR and HRMS.[11] The reactions using mesophilic enzymes were carried out at room temperature, pH 4.5 (*Aspergillus oryzae* 3.6mg), pH 5.0 (*Bacillus circulans* 3.6 mg) and pH 7.0 (*E. coli* 0.3 mg). The yield of N-acetyllactosamine and the synthesis/hydrolysis ratio have not been optimized according to the enzyme concentrations and reaction temperatures. [b] Hydrolysis was determined according to the free galactose content measured enzymatically by Hans-Otto Beutler method[12] using galactose dehydrogenase. [c] Yield was obtained by the analysis of transglycosylation reactions using an HPLC system equipped with a UV-visible detector (210 nm) and a LiChrospher NH_2 column (5μm, 150 × 4.6 mm I.D.). The column was eluted with isocratic 80% acetonitrile/water condition at a flow rate of 1.0 mL/min. The yield of N-acetyllactosamine was determined by use of calibration curves obtained from HPLC results

For preparative synthesis of N-acetyllactosamine, the reaction time is important since the secondary hydrolysis will begin to take place and the transglycosyl product will reach a maximum then start to decrease. The reaction time-course was followed for the production of N-acetyllactosamine in Gly001-07 and 09 catalyzed reactions (Figure 1).

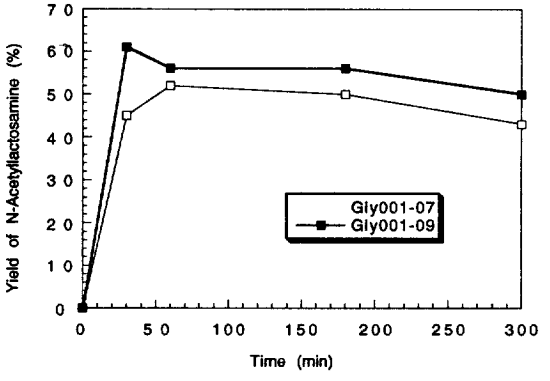


Fig. 1. Time-course of the production of N-acetyllactosamine with Gly001-07 and Gly001-09

It only took 30 min and 1 h for Gly001-09 and Gly001-07 to reach their optimum yields respectively. The reactions using enzymes Gly001-01, 02, 03, 04, 05 and 08 did not produce N-acetyllactosamine product. The specific activities of Gly001-01 and 05 were lower than the other 7 enzymes. With these two enzymes, no transglycosylation products were formed and only a small amount of starting material was hydrolyzed to galactose. Up to 92% hydrolyzed galactose was found in Gly001-02, 03, 04 and 08 catalyzed reactions. The observed transglycosylation product was mainly Galβ(1-6)GlcNAc.[11] For the conventional mesophilic β-galactosidases, no N-acetyllactosamine was formed with galactosidase from *Aspergillus oryzae* and *E. coli*. The galactosidase from *Bacillus circulans* was reported previously to catalyze the formation of N-

acetyllactosamine in high yield.[7] In this study, the yield of N-acetyllactosamine reached 25% in 30 min. After 5h, the secondary hydrolysis brought the yield down to 19% with the corresponding hydrolysis up to 71%. It is apparent that the CLONEZYME™ thermophilic glycosidases are more advantageous for the synthesis of N-acetyllactosamine in high yield. The results indicate that for high yield preparative organic synthesis, the commercial recombinant thermophilic enzyme libraries constitute a novel class of biocatalysts with unique specificities and robust properties more compatible with routine synthetic processes.

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