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Screening of strains and recombinant enzymes from *Thermus thermophilus* for their use in disaccharide synthesis

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ABSTRACT

Cell extracts from ten different strains of *Thermus thermophilus* have been screened for glycosidase activity. Among these, the sequenced strain HB27 hydrolyzed a wide variety of glycosides and increased six fold its β-glycosidase activity when grown with cellobiose in nutrient-limited media. We selected five genes encoding (putative) glycosidases (TTP0042, TTP0072, TTP0220, TTC0107 and TTP0222) from the genome of this strain, and the corresponding recombinant enzymes were overexpressed and purified. Several transglycosylation reactions using cellobiose-induced HB27 cell extracts and the purified recombinant enzymes were assayed. Biochemical properties and biosynthetic capabilities of the HB27 cell extracts and the TTP0042 enzyme were very similar, suggesting that this enzyme was responsible for most of the β-glycosidase activity detected in the HB27 strain. This was confirmed through the isolation and analysis of a null mutant of its encoding gene. With both, HB27 cell extracts and purified TTP0042 recombinant enzyme, we finally achieved high yields conditions for disaccharide production by transglycosylation with low amounts of self-condensed donor when high concentrations of a 1:5 donor:acceptor molar ratio was used.

1. Introduction

Oligosaccharides of glycoproteins and glycolipids play an important role in molecular interactions between cell membranes that are relevant for process such as embryogenesis, neuronal proliferation and apoptosis [1,2,3,4,5]. Thus, the synthesis of oligosaccharides requires a special control of the regio and stereoselectivity of the reaction that is difficult to achieve by chemical synthesis. For that reason the synthesis of oligosaccharides and glycoconjugates is often achieved by enzymatic methods, with glycosyltransferases, glycosidases and glycosynthases as biocatalysts [6–12]. Glycosidases (E.C. 3.2.1.) are enzymes whose natural function is the hydrolysis of glycosides. Reaction equilibrium of glycosidases can be controlled by competition of a glycoside acceptor in the active site of the enzyme against water molecules in order to develop synthesis of oligosaccharides and glycoconjugates. This type of reaction is known as transglycosylation, and it is used for oligosaccharide synthesis [13–15] through the appropriate control of the reaction conditions by the use of low water environment or activated glycosyl donors. An example for an important disaccharide in intercellular interactions is Gal-β(1→4)GlcNAc that constitutes the core of H2 Lewis antigen [16] and it is also well known as an specific substrate for *Streptococcus pneumoniae* β-galactosidase. Such enzyme could be related to the infection of human tissues by this bacterium because of its ability to deglycosylate *N*-linked glycans present on the surface of the cytoplasmic membrane [17–19].

The species *Thermus thermophilus* includes several strains of extreme thermophilic bacteria that constitute a potential source of thermostable and solvent-resistant enzymes, properties that could be required for the efficient synthesis of disaccharides if an appropriate glycosidase could be identified.

One of the main problems in enzymatic synthesis of disaccharide using β-glycosidases from *Thermus thermophilus* has been the self-condensation of the nitrophenyl donor used [20–24]. This have generated different strategies to improve disaccharide synthesis, including: directed evolution [25], glycosynthases design [26], acceptors engineering [27] and chemoenzymatic synthesis [28] to achieve specific sugars.

In this study we screened several strains of *Thermus thermophilus* for glycosidase activity against different substrates and characterized the biochemical properties from the best ones. We

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Table 1
Oligonucleotides used in this work. Letters in bold indicate restriction sites used for cloning of the amplified genes.

Name	Sequence (5′ > 3′)	Purpose
oTTP0042NdeI	AAA**CATATG**ACCGAGAACGCCGA	Forward, cloning TTP0042
oTTP0042HindIII	AA**AAGCTT**AGGTCTGGGCCCGC	Reverse, cloning TTP0042
oTTP0072NdeI	AAAA**CATATG**AGGCTGAACCTAGGA	Forward, cloning TTP0072
oTTP0072HindIII	AA**AAGCTT**ATAGAAGGGGGGCA	Reverse, cloning TTP0072
oTTP0220NdeI	AAAA**CATATG**CGGCTGGACCCCA	Forward, cloning TTP0220
oTTP0220HindIII	AA**AAGCTT**CTACTCCGCGAGAAGC	Reverse, cloning TTP0220
oTTP0222NdeI	AAAA**CATATG**AGGGTGGAGAAGGC	Forward, cloning TTP0222
oTTP0222HindIII	AA**AAGCTT**CACCAGGCCACCCT	Reverse, cloning TTP0222
oTTC0107NdeI	AAAA**CATATG**TGGTGGAAAGAGGCG	Forward, cloning TTC0107
oTTC0107HindIII	AA**AAGCTT**CTAGTCTAGCCGCACC	Reverse, cloning TTC0107
oMUT42EcoRIdir	AAAA**GAATTC**AAGGGCCTCGCCTTCT	Forward, mutant TTC0042
oMUT42HindIIIrev	AAAA**AAGCTT**CCCGTTTTCCGTGACG	Reverse, mutant TTC004

studied different strategies to improve their activities and evaluated their use as biocatalyst in glycoconjugate synthesis. Finally we cloned, expressed in *Escherichia coli* and in *Thermus thermophilus* HB27, and purified recombinant glycosidases from the strain HB27. The best glycosidase activities were analysed for their use in transglycosylation reactions.

2. Materials and methods

2.1. General

p-nitrophenol (*p*NP), *p*-nitrophenyl glycosides and bovine serum albumin (BSA), analytical standards of monosaccharides and disaccharides (D-(+)-galactose, D-(+)-fucose, D-(+)-mannose, D-(+)-glucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, Gal-(β1→4)-GlcNAc and Gal-(β1→6)-GlcNAc) for HPLC were purchased from Sigma–Aldrich; dye reagent for protein determination was purchased from Bio Rad. All other chemicals were from analytical grade. UV–visible spectra were recorded on a UV-2401 PC Shimadzu. HPLC Agilent 1100 with UV-vis detector using Mediterraneasea18 15 cm × 0.46 5 mm column (Teknokroma) with water: acetonitrile (75:25) as a mobile phase at a flow of 0.7 mL/min. HPLC Jasco with evaporative light scattering (ELSD), detector using NH2P50-4E amino column (Asahipak, Japan) with acetonitrile:water (80:20) as a mobile phase at a flow of 0.8 mL/min. NMR spectra were recorded on Bruker Avance 500 MHz spectrometer.

2.2. Bacterial strains, enzymes and growth conditions

Thermus thermophilus strains CC16, NR17, PRQ16, VG7, B, RQ1, PRQ25 and HN1.11 were a gift from Professor Milton da Costa. HB27 strain was a gift from Prof. Y. Koyama, and NAR1 strain was previously described [29]. *Thermus thermophilus* HB27nar is a derivative of HB27 that can grow anaerobically with nitrate as electrons acceptor [30]. *Escherichia coli* strains DH5α and BL21DE3 were used for gene cloning and protein overexpression, respectively. *Thermus thermophilus* strains were routinely grown in TB media [31], and *E. coli* was grown in LB. For plates, agar (1.5%, w/v) was added to these media. Ampicillin (100 mg/L) or kanamycin (30 mg/L) was added when required for plasmid selection. Transformation of *Escherichia coli* was carried out by standard methods and transformation of *Thermus thermophilus* was carried out by natural competence.

2.3. Screening of glycosidase activities in Thermus thermophilus strains

The above *Thermus thermophilus* strains were grown in TB media [31] under aerobic conditions for 16 h at 65 °C. Cells were harvested by centrifugation at 5000 × *g* for 10 min and suspended in phosphate buffer 30 mM pH 7.0. Cell concentration was analyzed with a Neubauer counting chamber. Glycosidase activity was determined using a continuous method as described below.

2.4. Effect of inducers in culture media over the glycosidase activity of Thermus thermophilus

To avoid putative repression by preferential catabolites, we used a nutrient-limited ¼ diluted TB medium to which we added 0.2% of saccharose, lactose, mannose, cellobiose, or melibiose. As reference control we used a parallel culture with similar concentration of glucose, which acts as preferential energy and carbon source in many bacteria, inhibiting the use of alternative sugars. Cells grown at 65 °C were harvested by centrifugation at 5000 × *g* for 10 min. Cells pellet was resuspended in phosphate buffer 50 mM, NaCl 50 mM, pH 7.30 and disrupted by sonication (three 30 s pulses, 300 MHz). Unbroken cells and insoluble debris were eliminated by centrifugation (14,000 × *g* for 15 min at 4 °C). Glycosidase activity was determined using a discontinuous method (see below) with identical amount of cell extract protein.

2.5. Enzyme assays and protein determination

Hydrolytic activities were determined by quantification of *p*NP liberated from the corresponding *p*NP-Glycosides. Reactions were carried out in a sodium phosphate buffer 50 mM pH 7.3 using either a continuous or a discontinuous method. Continuous method: increase in absorbance at 410 nm during 2.5 min at 65 °C in a 100 μL cell with 5 mM of the *p*NP-Glycoside. Discontinuous method: 200 μL reaction with 1.0 mM of the *p*NP-Glycoside at 80 °C for 10 min and stopping the reaction with1.00 mL of Na_2CO_3 0.20 M [20,32,33]. Protein concentration was determined by the Bradford method [34] with BSA (bovine serum albumin) as standard. One enzyme unit was defined as the amount of protein that hydrolyses 1 μmol of *p*NP-glycoside per minute under the conditions described above.

2.6. Cloning and overexpression of putative glycosydases from Thermus thermopohilus HB27

A bioinformatic search for putative glycosidases was carried out on the genome of *Thermus thermophilus* HB27 leading to the identification of five genes of codes TTP0042, TTP0072, TTP0220, TTP0222, and TTC0107. (http://wishart.biology.ualberta.ca/BacMap/). Every gene was amplified by PCR (Polymerase Chain Reaction) with the corresponding pair of plasmids (Table 1) that included sites for the enzyme of restriction NdeI at the 5′ end and HindIII at the 3′ end. The amplified genes were inserted into the equivalent restriction sites of plasmid pET28b+ (Novagen) from which they can be produced as N-terminal six-histidines tagged proteins. For overexpression of the corresponding proteins, cells of *E. coli*