Improvement of the Efficiency of Transglycosylation Catalyzed by α-Galactosidase from *Thermotoga maritima* by Protein Engineering

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**Abstract**—At high concentrations of p-nitrophenyl-α-D-galactopyranoside (pNPGal) as a substrate, its hydrolysis catalyzed by α-galactosidase from *Thermotoga maritima* (TmGalA) is accompanied by transglycosylation resulting in production of a mixture of (α1,2)-, (α1,3)-, and (α1,6)-p-nitrophenyl (pNP)-digalactosides. Molecular modeling of the reaction stage preceding the formation of the pNP-digalactosides within the active site of the enzyme revealed amino acid residues which modification was expected to increase the efficiency of transglycosylation. Upon the site-directed mutagenesis to the predicted substitutions of the amino acid residues, genes encoding the wild type TmGalA and its mutants were expressed in *E. coli*, and the corresponding enzymes were isolated and tested for the presence of the transglycosylating activity in synthesis of different pNP-digalactosides. Three mutants, F328A, P402D, and G385L, were shown to markedly increase the total transglycosylation as compared to the wild type enzyme. Moreover, the F328A mutant displayed an ability to produce a regio-isomer with the (α1,2)-bond at yield 16-times higher than the wild type TmGalA.

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Carbohydrate structures (glycostructures), such as glycoconjugates or oligo- and polysaccharide fragments, play numerous and often not fully understood roles in cellular processes. Glycostructures are involved in inflammation, intercellular interactions and signaling, immune response, viral and bacterial infections, energy accumulation, and in various matrix processes [1-3]. Natural glycostructures are characterized by a great variety, but to study and understand their functions caused by the structural variability it is necessary to have significant amounts of the compound under study. Moreover, multiple biological activities of glycostructures determine the wide application of oligosaccharides with the desired composition in medicine and biotechnology [4-7]. Traditional chemical approaches for synthesizing glycostructures consist of many time-consuming stages including procedures of blocking and deblocking of reactive chemical groups. Such syntheses, in addition to the desired product, give ecologically harmful waste. But for chemical synthesis of various carbohydrate-containing molecules there is an alternative, namely, enzymatic synthesis using two classes of enzymes, glycosyltransferases and glycoside hydrolases. However, the high price of nucleotide-activated sugars used as substrates for the directed synthesis of oligosaccharides with glycosyltransferases makes these enzymes less attractive. In turn, glycoside hydrolases capable of transglycosylating (transferring a carbohydrate substrate (donor) residue bound to the active site onto the hydroxyl of another sugar or alcohol (acceptor)) are promising tools for producing oligosaccharides [8]. As a rule, these enzymes are stable, can be easily isolated, and their substrates are rather available. Glycoside hydrolases have already been used for synthesis of carbohydrate-containing structures for several dozens of years. Hundreds of papers concerning the synthesis of the glycosidic bond using glycoside hydrolases have been published (reviews [9-12]).

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\( \alpha-D \)-Galactosidases (EC 3.2.1.22) are glycoside hydrolases cleaving off the terminal residues of \( \alpha-D \)-galactose from the non-reducing end of \( \alpha-D \)-galactooligosaccharides [13] with retention or inversion of the anomeric configuration of the substrate C1 atom in the product (the so-called “retaining” or “inverting” glycoside hydrolases [14]). In some cases “retaining” \( \alpha-D \)-galactosidases display a transglycosylating activity, i.e. the enzyme is able not only to hydrolyze but also to synthesize galactose-containing compounds [15] required in different fields of biotechnology, pharmaceutics, and medicine. More often, \( \alpha-D \)-galactosidases have a low specificity in the +1 subsite of the active site, and that is why many “retaining” \( \alpha-D \)-galactosidases can synthesize virtually the whole spectrum of galactosidic bonds [16-21].

The gene of \( \alpha-D \)-galactosidase from *Thermotoga maritima* (TmGalA) used in our work was sequenced and cloned in *E. coli* [22]. Based on comparative analysis of the amino acid sequences, this enzyme was classified to family 36 of glycoside hydrolases (http://www.cazy.org/ghasea.html [23]). Due to biochemical features of TmGalA (the thermostability and transglycosylating activity in the presence of high concentrations of the acceptor), this enzyme seems promising for synthesis of \( \alpha-D \)-galactose-containing structures.

The aim of this work was to establish the possibility of managing the transglycosylating activity of TmGalA. The paper presents theoretical calculations and results of experimental assay that have confirmed that the +1 subsite contains amino acid residues whose substitution can result in a significant increase in the transglycosylating activity of the \( \alpha-D \)-galactosidase.

**MATERIALS AND METHODS**

Molecular modeling. Molecular modeling was performed using the Molsoft ICM Pro 3.6 program [24]. The known crystal structure of TmGalA (PDB code 1ZY9) was taken as an initial model for the rational design. The position of the \( \alpha-D \)-galactopyranose (\( \alpha \)-Galp) binding site in the catalytic center of TmGalA was determined by spatial superposition of this enzyme structure with structures of homologous \( \alpha-D \)-galactosidases containing \( \alpha \)-Galp in the active site and isolated from *Oryza sativa* (PDB code 1UAS; family 27 [25]) as well as from *Lactobacillus acidophilus* (PDB code 2XN2; family 36 [26]). Then the \( \alpha \)-Galp orientation in the catalytic center of TmGalA was refined by flexible molecular docking (a flexible ligand and mobile side chains of the receptor amino acids) that allowed us to choose the sugar conformation in the active site with the position and orientation coinciding with \( \alpha \)-Galp in two homologous enzymes. Then a galactosyl–enzymatic complex was created: the OH1 group of galactose was removed, and its anomic atom C1 was artificially covalently bound to atom O\( \delta 1 \) or O\( \delta 2 \)
of the side chain of the enzyme D327 residue acting as a nucleophile. Positions of water molecules obtained from crystal structure were taken into account only during structural optimization of the resulting intermediate (MMFF94 force field [27] was used). Mutant forms of this intermediate were modeled by replacement of target amino acids, followed by spatial optimization of the mutated side chain and its neighbors within 5 Å. The optimized models were used for flexible docking with \( p \)-nitrophenyl \( \alpha-D \)-galactopyranoside (\( p \)NPGal) to evaluate possible mutual orientations of the intermediate (\( \beta \)-galactosyl–TmGalA) and the acceptor (\( p \)NPGal) located in subsites –1 and +1, respectively.

Conformations of \( p \)NPGal and galactose molecules were initially analyzed in the MM+ force field using a molecular mechanics approach. The conformations of molecules having the minimal free energy were subjected to geometry optimization in the PM3 force field using the Polak–Ribiere algorithm with the energy gradient vector accuracy of 0.01 kcal/mol. These calculations were performed using the HYPERCHEM 8.0 program. Partial charges resulting from the optimization procedure were assigned to the ligand atoms prior to the docking procedure. During the docking, the sugar ring in \( p \)NPGal retained the “chair” conformation. Three independent runs of molecular docking, starting from different initial spatial location of \( p \)NPGal in the vicinity of the enzyme active center, were applied.

**Strains and plasmids.** All chemicals were obtained from Sigma-Aldrich or Acros Organics unless otherwise noted. \( p \)NPGal was synthesized from D-galactose as described for a glucoside [28]. A plasmid with the wild type \( \alpha-D \)-galactosidase gene was kindly donated by Prof. Robert Kelly (North Carolina State University, USA). The \( \alpha-D \)-galactosidase gene (\( galA \)) was cloned from the thermophilic bacterium *Thermotoga maritima* MSB8 (ORF TM1192) [22]. The pET24D vector (Novagen, USA) containing the \( \alpha-D \)-galactosidase gene was isolated using QIAGEN plasmid midi kit (cat. No. 12143). Site-directed mutagenesis was carried out and the sequence was confirmed by Evrogen (http://www.evrogen.com/), yielding seven plasmids with point mutations P402D, P402S, G385L, F328A, L195C, F194K, and W85Y. Native and mutant \( galA \) genes on these plasmids were expressed in *E. coli* BL-21(DE3) [29], and the recombinant enzymes were purified from cells of culture grown overnight as previously described [22].

**Analysis of hydrolytic activity of the wild type TmGalA and its mutants.** The kinetics of hydrolysis of \( p \)NPGal by the wild-type (wt) and mutant TmGalA were measured at 37°C in 50 mM sodium phosphate buffer, pH 5.0. One unit of the activity was defined as amount of the enzyme releasing 1 \( \mu \)M of nitrophenol from \( p \)NPGal per minute. Reaction mixtures (total volume of 50 \( \mu \)l) were pre-equilibrated, and the reaction was initiated by the addition of 10 \( \mu \)l of enzyme solution (0.02-0.4 \( \mu \)g/\( \mu \)l in wt or mutant