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Role of Glu445 in the substrate binding of β-glucosidase

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ABSTRACT

A previously uncharacterized gene in *Neosartorya fischeri* was cloned and expressed in *Escherichia coli*. It was found to encode a β-glucosidase (NfBGL1) distinguishable from other BGLs by its high turnover of *p*-nitrophenyl β-D-glucopyranoside (*p*NPG). Molecular determinants for the substrate recognition of NfBGL1 were studied through an initial screening of residues by sequence alignment, a second screening by homology modeling and subsequent site-directed mutagenesis to alter individual screened residues. A conserved amino acid, E445, in the substrate binding pocket of wild-type NfBGL1 was identified as an important residue affecting substrate affinity. Replacement of E445 with amino acids other than aspartate significantly decreased the catalytic efficiency (k_{cat}/K_m) of NfBGL1 towards *p*NPG, mainly through decreased binding affinity. This was likely due to the disruption of hydrogen bonding between the substrate and the carboxylate oxygen of the residue at position 445. Density functional theory (DFT) based studies suggested that an acidic amino acid at position 445 raises the substrate affinity of NfBGL1 through hydrogen bonding. The residue E445 is completely conserved indicating that this position can be considered as a crucial determinant for the substrate binding among GHs tested.

1. Introduction

β-Glucosidases (BGLs) are exo-type glycoside hydrolases (GHs) that cleave β-glucosidic bonds from the nonreducing ends of their substrates [1]. A number of genes that encode cellulases, including BGLs, have been cloned and expressed in both *Escherichia coli* and *Saccharomyces cerevisiae*, but their enzymological properties, especially structure-function relationships, have not been fully characterized [2]. BGLs play important roles in a variety of biological and biotechnological processes [3,4].

Families of GHs have been classified within super families or clans based on amino acid sequence similarity [5]. Family 1 is included in clan GH-A (also named superfamily 4/7). Its members are characterized by an 8-fold α/β barrel structure, in which two amino acids of the active site are directly involved in the catalysis: the acid/base catalyst and the nucleophile are located in β strands number 4 and 7, respectively [6,7]. Although different in quaternary structure, the monomeric form of GH1 enzymes is composed of a single-domain $(\beta/\alpha)_8$ barrel with a molecular mass of ca. 50 kDa [8]. The hydrolysis of the β-glycosidic bond occurs through a double-displacement catalytic mechanism [9] that retains the conformation of the anomeric carbon, with two conserved glutamate residues acting as nucleophile and acid/base. GH1 enzymes have many common structural features in their catalytic sites, despite having activity towards a large variety of substrates. Even minor changes in the substrate structure can significantly alter the enzymatic behavior [8].

Although family 1 BGLs are structurally and mechanistically uniform, the amino acid residues involved in substrate binding and stabilizing enzyme–substrate complexes may differ chemically or in their positioning within the active site [10]. Structural studies have been performed to elucidate the structure-function relationship of BGLs [11,12]. For example, Masayuki et al. [13] reported the importance of E191 and E407 in catalysis through site-directed mutagenesis. Nonetheless, detailed analysis of molecular determinants controlling the substrate affinity of BGLs is still lacking.

This work reports a BGL (NfBGL1), a GH1 enzyme, cloned and characterized from *N. fischeri* NRRL181, which demonstrates a high turnover of *p*-nitrophenyl β-D-glucopyranoside (*p*NPG). A 3D model of NfBGL1 was constructed using the crystal structure of *P. chyrsosporium* β-glucosidase A (PcBGLA) as the template. The

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molecular dynamics (MD) simulation studies reveal several key residues that interact with the substrate. The amino acid E445 was found to interact with the substrate through hydrogen bonding. The site-directed mutagenesis, MD, and density functional theory (DFT) studies reported here suggest that E445 contributes to the substrate binding of a BGL (NfBGL1), a GH1 enzyme.

2. Materials and method

2.1. Materials, strains and culture condition

Reagents for PCR by Ex-*Taq* DNA polymerase were purchased from Promega (Madison, WI). Restriction enzymes were obtained from New England Biolabs (Ipswich, MA). Plasmid isolation kit, oligonucleotide primers (Bioneer, Daejeon, South Korea) and pET28a expression vector (Novagen, Madison, WI) were purchased. A Ni-nitrilotriacetic acid Superflow column was used for purification (Qiagen, Hilden, Germany). Electrophoresis reagents were from Bio-Rad, and chemicals for assay were from Sigma–Aldrich (St. Louis, MO). A plasmid containing wild-type (WT) NfBGL1 gene was used for the production of WT NfBGL1 protein. *E. coli* DH5α and *E. coli* BL21 (DE3, codon plus) hosts were used to transform and to express the proteins, respectively. Cultures of *E. coli* carrying WT and mutated genes of NfBGL1 were grown for protein expression in Luria-Bertani (LB) medium supplemented with kanamycin (25 μg ml^{-1}) and chloramphenicol (50 μg ml^{-1}) at 37 °C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to the culture medium at a finial concentration of 1 mM, and incubation continued with shaking at 16 °C for 18 h.

2.2. Cloning and expression of NfBGL1

RNA was extracted from frozen mycelia using an RNeasy Plant Mini Kit (Qiagen, CA, USA) following the manufacturer's instructions. Full-length cDNA was amplified by RT-PCR using a Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, CA, USA). The *bgl* gene was amplified by PCR from *N. fischeri* cDNA using two oligonucleotide primers, 5′-TTTGGATCCCGATGGACCTGCAATCCGTTC-3′ (*Bam*HI restriction site is underlined) and 5′-GCGGTCGACTTAAGAGTCCTTGATCAA-3′ (*Sal*I restriction site is underlined). *Bam*HI and *Sal*I sites were incorporated into the forward and reverse primers for cloning into the expression vector pET28a. The recombinant plasmid was then transferred into *E. coli* BL21 (DE3, codon plus), and recombinant enzyme was expressed using 1 mM IPTG at 16 °C for 18 h. The induced cells were harvested by centrifugation at 4 °C for 15 min at 10,000 × *g*, rinsed with phosphate-buffered saline, and stored at −20 °C.

2.3. Site-directed mutagenesis of NfBGL1

Site-directed mutagenesis was carried out using a QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Recombinant plasmid *p*ET28-NfBGL1 containing the WT NfBGL1 gene was used as a DNA template. Plasmids containing the correct mutant genes were then used to transform *E. coli* BL21 (DE3, codon plus). Colonies selected by kanamycin and chloramphenicol resistance were used for protein expression.

2.4. Protein purification and quantification

Cell pellets were resuspended in 20 mM sodium phosphate buffer (pH 7.5). The cell suspension was incubated with 1 mg ml^{-1} lysozyme on ice for 30 min. Cells were disrupted by sonication at 4 °C for 5 min and the lysate was centrifuged at 14,000 × *g* for 20 min at 4 °C to remove cell debris. The resulting crude extract was retained for purification on a Ni-nitrilotriacetic acid Super flow column (3.4 by13.5 cm; Qiagen) previously equilibrated with a binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). Unbound proteins were washed from the column with a washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 150 mM imidazole, pH 8.0). Protein was then eluted from the column with an elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Enzyme fractions were analyzed by sodium dodecyl sulfate- 10% polyacrylamide gel electrophoresis (SDS-10% PAGE) and visualized by Coomassie brilliant blue R250 staining. Protein concentration was determined by measuring absorbance at 280 nm (ε = 10.7 cm^{-1} mM^{-1}) using a Varian Cary 100 Bio UV–vis spectrophotometer (Palo Alto, CA).

2.5. Enzyme assay and determination of kinetic parameters

BGL activity was assayed using *p*NPG as substrate. 1 ml enzymatic reaction mixtures containing 100 μl enzyme solution and 10 mM *p*NPG (final concentration) in 100 mM sodium acetate buffer (pH 5.0) were incubated for 15 min at 50 °C [14]. Released *p*-nitrophenol was measured by absorbance (A_{415}, ε_{415} = 17.0 mM^{-1} cm^{-1}) after the addition of 2 M Na_2CO_3. Kinetic parameters of NfBGL1 were determined through incubation with 1–400 mM *p*NPG in 100 mM potassium phosphate buffer at pH 6.0 and 40 °C. K_m and V_{max} were determined by the Michaelis-Menten method using Prism 5 software (Graphpad Software, Inc., CA, USA). One unit of *p*NPG hydrolyzing activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute. The optimal pH of NfBGL1 activity was determined by incubating the purified enzyme at 40 °C for 15 min in different buffers: sodium acetate (100 mM, pH 3–6), potassium phosphate (100 mM, pH 6–8), and glycine (100 mM, pH 8–10). Optimal temperature was determined by incubating the enzyme in potassium phosphate buffer (100 mM, pH 6) for 15 min at temperatures between 20 and 70 °C. Substrate specificity of NfBGL1 towards naturally and artificially occurring substrates were studied as previously reported [15].

2.6. Homology modeling

Three-dimensional homology models of the WT and mutant proteins were generated using the Build Homology Models (MODELER) module in Discovery Studio 2.5 (DS 2.5, Accelrys Software Inc., San Diego, CA). The crystal structure of PcBGLA (PDB accession code 2E3Z) was used as template. Comparative modeling generated the most probable structure of the query protein through alignment with template sequences, while simultaneously satisfying spatial restraints and local molecular geometry [16]. The model sequences' fitnesses in their current 3D environment were evaluated by Profiles-3D Score/Verify Protein (MODELER) as implemented in DS 2.5. The discrete optimized protein energy (DOPE) score in MODELER was also calculated to assess the protein structures' quality. Root mean square deviations (RMSD) between the models and template were calculated by superimposing their crystal structures. The evaluated 3D models were used for docking and post docking analysis. Hydrogen atoms were first added to the 3D models. They were minimized to have stable energy conformations relieved from close contacts. Different substrate molecules were docked into the substrate binding pockets (SBPs) of NfBGL1 and the mutant models using C-DOCKER, an MD-simulated annealing-based algorithm module in DS 2.5 [17], with different poses then created using random rigid-body rotations and simulated annealing. Before docking, the structures of the protein, substrate and their complexes were minimized for energy using the CHARMm [18] forcefield as implemented in DS 2.5. A full potential final minimization was then used to refine the substrate (ligand) poses. The substrate orientation with lowest interaction energy was chosen for subsequent docking studies. Based on the C-DOCKER energy, the docked conformation of the substrate was retrieved for post-docking analysis [19].

2.7. Thermodynamics of substrate binding

The generalized gradient approximation, within DFT formalism, was used as implemented by the DMol^3 module of the Materials Studio software (Accelrys Inc., San Diego). Geometry optimization calculations were performed using the Becke-Lee-Yang-Parr (BLYP) exchange correlation functional and the double numerical with polarization (DNP) basis set, the best available set in DMol^3 [20]. This basis set considers a polarization, *d* function, on heavy atoms and a polarization, *p* function, on hydrogen atoms. It is comparable to the split-valence double-ζ6-31G in size; however, it is more accurate than the Gaussian basis sets of the same size [21]. The BLYP/DNP theory level has been demonstrated adequate in several studies [22], with errors expected to be in the second decimal place of calculated bond lengths (angstroms) and in the order of 2–5 kcal/mol in energies. Solvation effects of the part of the enzyme not included in the model cluster were estimated considering a homogeneous polarizable medium with a continuum solvation model–COSMO (conductor like screening model), implemented in the DMol^3 module. This model has solute molecules that form cavities within the dielectric continuum of permittivity that represents the solvent. The dielectric constant, ε, was set at 4, the standard used in modeling protein surroundings. Geometry optimizations were performed in the presence of this solvation model, allowing final energies to include the DMol^3/COSMO electrostatic energy [21].

2.8. Analytical methods and protein database search

Optical spectra were recorded on a Varian Cary 100 Bio UV–vis spectrophotometer (Palo Alto, CA). CD experiments were performed on a Jasco J-815 spectrophotometer (Jasco corp., Tokyo, Japan) at 20 °C. Spectra were recorded between 190 and 300 nm at 100 nm/min. CD spectra were base line corrected and the measured ellipticities are expressed in mdeg. Amino acid sequences deduced from the *BGL* gene sequences of *N. fischeri* were compared with those of related enzymes from other sources using the BLAST network at the National Center for Biotechnology Information. Multiple sequence alignment was performed with the ClustalW program.

3. Results

3.1. Cloning and characterization of NfBGL1

A previously uncharacterized gene (nucleotide ID: 4589708), thought to encode a β-glucosidase (NfBGL1), was cloned from *N. fischeri* and its nucleotide sequence was determined. DNA sequence analysis found an open reading frame of 1467 bp. The deduced BGL gene product showed ∼50% amino acid identity with the GH1