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Effects of mutations of non-catalytic aromatic residues on substrate specificity of *Bacillus licheniformis* endocellulase *cel12A*

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ABSTRACT

Cel12A, a gene that encodes an endocellulase of *Bacillus licheniformis*, was cloned and overexpressed in *E. coli* DE3. The rBLCel (wild-type recombinant cellulase) enzyme was assayed and characterized for its physical properties, including its optimum pH (9.0), temperature (50 °C), thermostability (175 h) and kinetic parameters (K_m16.38 ± 0.550 mg ml^{-1}; k_{cat} 134.91 ± 0.377 min^{-1} and k_{cat}/K_m8.23 ± 0.026 ml min^{-1} mg^{-1}). To determine the role of conserved amino acids in the substrate binding pocket, five single mutations and one double mutation were studied for their effects on substrate specificity. All six purified engineered enzymes were assayed and compared to rBLCel regarding their catalytic activity with a specific substrate (CMC). All of the recombinant enzymes (wild type and engineered) were also assayed with non-specific substrates, such as xylan, avicel and filter paper (FP). Out of five point-mutant enzymes, three mutants (W197A, W98A and Y89A) showed reduced specific activity compared to rBLCel. However, the W139A and W53A mutants exhibited a 67% and 4% relative increase in their specific activity with a specific substrate (CMC), respectively. After replacing aromatic residues, the affinity towards the CMC increased in the case of the W139A and W53A mutants, indicating that the aromatic residues play a key role in substrate affinity and binding. One of the significant results that we obtained was that the increase in activity was additive in the double mutant enzyme, W139A_W53A, which showed 1216% higher activity than rBLCel on filter paper (FP). The activity of all of the mutated enzymes with non-specific substrates indicates that the substrate binding pocket is altered and has become flexible toward other substrates after mutation. Moreover, given the importance of the two enzymes in degrading lignocellulose, one bifunctional enzyme endowed with the two catalytic activities may be of great advantage. Despite the broad spectrum of cellulases being isolated, no single enzyme is completely suitable as it is, for the hydrolysis of lignocellulose. These enzymes have been used due to their biotechnological applications in several industries, including bio pulping wood, treatment of animal feed to increase digestibility, agro-processing, juice processing and baking.

1. Introduction

Global demand for energy has grown with the development of new industries. This demand has necessitated constant improvement and a ceaseless search for new sources. Lignocellulosic waste is a potential source of renewable energy. Complete hydrolysis of lignocellulose requires many lignocellulase enzymes. These hydrolytic enzymes have similar mechanisms of action that catalyze cleavage of b-1,4 glycosidic bonds in biomass polymers [1]. Though many of these enzymes are bifunctional in nature [2], the efficiencies of hydrolysis of xylan by cellulase and cellulose by xylanase are very poor. Binding of a ligand to a specific position of an enzyme for a specific reaction mainly depends on the amino acid residues present in the enzyme binding site [3]. These residues permit a specific substrate to bind. Many multifunctional enzymes exist; these enzymes are widely distributed in nature and have multiple activities against different substrates of same group [4]. During evolution, these enzymes have become increasingly specific toward their substrate based on availability [5,6]. A closer look at the substrate-binding region of these enzymes shows that the orientation of amino acids in the binding pocket determines the substrate preference. Thus, there is a need to modify and improve enzymes to make them potent, efficient and suitable for complete hydrolysis. This could be

Abbreviations: rBLCel, wild-type recombinant cellulase enzyme; MTCC, Microbial Type Culture Collection; LB, Luria-Bertani; k_{cat}, turnover number; K_m, Michaelis constant; PDB, Protein Data Bank; V_{max}, maximum velocity; *cel12A*, endocellulase gene; CMC, Carboxymethylcellulose; FP, Filter paper; ITASSER, Iterative Threading ASSEmbly Refinement

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done by altering the substrate specificity of enzymes to help accommodate various substrates of the same group. Approaches such as enzyme engineering, reconstitution of enzymes and bioprospecting for superior enzymes are gaining importance [7]. With the use of site-directed mutagenesis, the role of different amino acids within the catalytic domain of an enzyme can be studied [8].

In this study, mutants were created to answer the following questions: a) Are aromatic residues involved in substrate binding? b) Do aromatic residues function as gating residues? c) Are aromatic/non-catalytic residues involved in altering substrate specificity?

This study also aims to broaden the catalytic properties and alter the substrate specificity of the selected cellulase enzyme. Five different aromatic residues (W197, W139, W98, W53 and Y89) in the substrate binding pocket of CEL12A were found to be highly conserved, and these aromatic amino acids were targeted for substitution with alanine (A) through site-directed mutagenesis. Wild-type *cel12A* and its mutated genes were cloned and overexpressed in *E. coli* BL21 (DE3). All recombinant enzymes were studied for their biochemical properties.

2. Materials and methods

2.1. Bacterial strains and plasmid

Bacillus licheniformis 429 was procured from MTCC (Microbial Type Culture Collection), Chandigarh, India. The culture was revived on nutrient medium at 37 °C. For cloning and expression, *E. coli* (DH5α) and *E. coli* BL21 (DE3) (Invitrogen) were used, respectively. The *E. coli* strains were grown on LB (Luria-Bertani) medium. The pET101/D-TOPO (5753 bp) vector was used (Invitrogen, Waltham, Massachusetts, USA). Blunt-end PCR products were used for directional cloning using a Champion™pET Directional TOPO® Expression Kit (Invitrogen, USA).

2.2. Cloning, expression, purification and characterization of rBLCel

Genomic DNA was isolated from *Bacillus licheniformis* 429 using the standard method [9]. Primers were synthesized for the *cel12A* gene (IDT, USA): 1) *cel12A*_FP (CACCATGAAAAACAACCATTTGCTA) and *cel12A*_RP (GCGGACCGTTACGTCCCAATT). Polymerase chain reaction contained 0.4 µM forward and reverse primers, 2.0 µl of template DNA, 2.5 µl of 10X high fidelity PCR buffer, 2.0 mM $MgSO_4$, 0.2 mM deoxyribonucleotide solution mix, and 1 U of high fidelity platinum Taq DNA polymerase. The reaction mix was brought up to a volume of 25 µl by Milli-Q water. Amplification started with an initial denaturation at 94 °C for 2 min, denaturation for 15 s, followed by annealing at 60.2 °C for 30 s, extension for 1 min at 68 °C and final extension was for 10 min at 68 °C. The amplified product was purified and used as an insert for cloning in the pET101/D-TOPO vector. The ligated product was transformed using the standard method of chemical transformation. A recombinant clone (rBLCel) was expressed in *E. coli* BL21 (DE3) cells by growing them while shaking at 37 °C in 100 µg ml^{-1} ampicillin-containing AIM-Super growth medium (alternate for IPTG-inducible expression) with trace elements (Hi-Media). Overexpressed recombinant enzyme was purified in a single step using Ni-NTA (Nucleo-pore, Genetix, India) affinity chromatography. The concentration of purified protein was determined using the Bradford assay [10]. The apparent molecular weight of the purified protein was also estimated using polyacrylamide gel electrophoresis (SDS-PAGE) on 1.0 mm slab gel of 12% polyacrylamide (w/v) [11].

The rBLCel enzyme activity was determined using a reaction mixture containing 190 µl of 2% *w/v* CMC (Carboxymethylcellulose, Sodium salt medium viscosity 200–300 CPS) prepared in 0.2 M glycine-NaOH buffer (pH 9.0), 13 µg (10 µl) of diluted enzyme and 100 µl of buffer (Gly-NaOH, pH 9.0). The reaction system was incubated at 50 °C for 60 min and shaken at 10 min intervals. Released sugars were estimated using the DNS (3,5-dinitrosalicylic acid) method [12]. One unit (U) of endocellulase activity was defined as the amount of enzyme required to release 1 µmol of glucose (reducing sugar) in 1 min under the mentioned conditions. The characteristics of the rBLCel enzyme, such as pH, temperature, stability at maximum pH and temperature, kinetic parameters, effect of metal ions and detergents on enzyme activity and bi-functionality, were tested.

2.2.1. rBLCel characterization

pH: pH ranges from 3.0–5.0, 6.0–8.0 and 9.0–13.0 were made using Citrate Buffer (0.1 M), potassium phosphate buffer (0.1 M) and Glycine-NaOH buffer (0.1 M), respectively. Buffers were prepared according to the standard methods of biochemical analysis [13]. The effect of pH was determined using a pH range of 3.0–13.0 at 50 °C. **Temperature:** The effect of temperature on rBLCel was determined by incubating the reaction mixture with enzyme at a temperature from 10 °C to 100 °C. The reaction mixture contained 0.1 M glycine-NaOH buffer at pH 9.0 and 2% *w/v* CMC as a substrate. **The effect of metal ions and detergents** on rBLCel activity was determined with a 5 mM concentration of various effectors. Metal ions, chemicals and detergent, such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+}, K^{+}, cd^{2+}, Ni^{2+}, Pb^{2+}, Na^{+}, Li^{2+}, SDS (sodium dodecyl sulfate), PVP (poly vinyl pyrrolidone), Triton 100 and Tween 20, were prepared. Solutions of salts/detergents were mixed with the enzyme and were incubated at room temperature for 60 min. CMC (2% *w/v*) was then added, and the reaction mixture was incubated for 60 min to allow the reaction to proceed. The activity difference compared to rBLCel was measured under optimum conditions at pH 9.0 and 50 °C. **Stability:** The stability of rBLCel was evaluated by incubating the enzyme at 50 °C with and without 0.1 M Gly-NaOH buffer, pH 9.0. For thermostability, the enzyme was incubated without any buffer. In the case of pH stability, the enzyme was incubated with buffer at pH 9.0. At different time intervals, the incubated enzyme was incubated with 2% *w/v* CMC for 60 min under standard conditions. The amount of reducing sugar was estimated by stopping the reaction by addition DNSA reagent. To determine the **catalytic properties** of rBLCel, a substrate saturation curve was plotted. The kinetic parameters, such as K_m, k_{cat} and k_{cat}/K_m, were determined by a Line Weaver-Burk plot according to the MM equation. The bi-functionality of rBLCel was also checked using a non-specific substrate, such as beechwood xylan (Sigma-Aldrich); Whatman filter paper; and avicel (pH-101, Sigma-Aldrich).

2.3. Sequence analysis and site-directed mutagenesis

The sequence study was carried out using the *cel12A* gene (787 bp) of *Bacillus licheniformis* 429 as the template. Online software and tools, EMBOSS, BLAST (ExPASy), and ClustalW2 (EBI) were used for nucleotide sequence analysis while Compute pI/Mw (ExPASy) and AACompIdent (ExPASy) were used for protein sequence analysis. Using the multi-stage mapping algorithm FTmap, we identified potentially favorable binding positions. Sets of various probable binding residues were identified for single mutations based on homology modeling of the available protein structures in the Protein Data Bank (RCSB PDB). In the *cel12A* gene, site-specific substitutions were designed and mutated fragments were generated using pairs of primers (Table 1). Full-length *cel12A* mutated genes were obtained by overlapping the mutated fragments using the overlap extension PCR method [14,15], and a library was generated.

2.4. Characterization of mutated enzymes and its substrate specificity

Mutated amplicons were ligated to the pET101/D-TOPO vector and the clones were transformed. All of the mutated recombinant clones of rBLCel (W197A, W139A, W98A, W53A, Y89A and W53A_W139A) were expressed in *E. coli* BL21 (DE3). The overexpression and purification methods were the same used for the wild-type recombinant enzyme (rBLCel). Overexpressed enzymes were purified and characterized for the optimum pH, temperature, stability of enzymes and kinetic