Journal of Bioscience and Bioengineering
VOL. 115 No. 6, 618–622, 2013

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Alkalophilic adaptation of XynB endoxylanase from *Aspergillus niger* via rational design of p*K*a of catalytic residues

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Received 10 September 2012; accepted 5 December 2012
Available online 4 January 2013

Based on the strategy of changing pH-stability profiles by altering p*K*a values of catalytic residues, rational protein engineering was applied to improve alkalophilic adaptation of *Aspergillus niger* endoxylanase XynB. Computational predictions and molecular modeling were carried out using PROPKA server and SWISS-MODEL server, respectively. Three endoxylanase mutant of S108V, N151E, and Q178R, in which the p*K*a values of either catalytic glutamate residues shifted, were generated. In agreement with expectation, the variant of Q178R improved alkalophilic performances. The mutant Q178R raised the optimum pH of XynB from 5.5 to 6.0 and retained 37% of the maximum activity at pH 8.0. Interestingly, the p*K*a values of Glu84 and Glu175 in Q178R are 7.91 and 6.32, respectively. The p*K*a of Glu175 is lower than that of Glu84, as opposed to the fact that the p*K*a of Glu84 is lower than that of Glu175 in other GH11 xylanases. It indicated that Glu175 may convert into a nucleophile residue and Glu84 into an acid/base residue.

[**Key words:** Endoxylanase; Alkalophilic adaptation; Rational design; *Aspergillus niger*; pH-stability]

Xylan, the second most abundant renewable resource after cellulose, is the main component of hemicellulose and is composed of β-1,4-linked xylopyranose chains with 4-*O*-methyl-D-glucuronic acid, arabinose, *O*-acetyl, and uronic acids substituent (1). Its complete hydrolysis requires a large variety of enzymes among which endo-β-1,4-xylanases are preponderant. These enzymes have been mainly classified into two families, F/10 and G/11, based on hydrophobic cluster analysis and sequence homology (2). Family F/10 are endo-β-1,4-xylanses with higher molecular mass than family G/11 xylanses, and presenting (α/β)8 barrel folds in three-dimensional (3D) structure (3). Family G/11 are xylanases with lower molecular masses (<30 kDa) (4) and are encoded as precursors composed of a signal peptide and a mature xylanase. The 3D structures of family G/11 xylanases have the overall shape of right hand (5).

Xylanases have attracted an increased interest because of their industrial usage, including paper and pulp industries, food and feed industries. One of the major current applications of xylanases is in paper industry where the use of these enzymes decreases the amount of hazardous bleaching substances used in the delignification process, and also results in a better pulp brightness (6,7). The industrial pulping is usually carried out at a high-alkaline pH environment (8), hence requiring xylanases to be operationally stable under such conditions. However, the great majority of xylanases reported so far are neither active nor stable at both high temperature and high pH. In recent years, a growing number of endo-xylanases has been isolated from, in particular, extremophilic microorganisms to meet increasing industrial demands for enzymes that can cope with the often harsh conditions of biotechnological processes (9). In addition, numerous protein engineering endeavors have been directed toward improvement of their stability and/or optimal activity at elevated temperatures and/or extreme pHs. Real rational pH-engineering were the creation of a more alkalophilic xylanase by computational model (10), the pHopt increase of xylanase B from *Thermotoga maritima* by five amino acids substitution (11), and the improvement of alkalophilic effects of *Streptomyces* and *Bacillus* xylanase (12,13). But there is little report about rational alkalophilic engineering of F/11 xylanase from *Aspergillus niger*.

Filamentous fungus *A. niger* produces a broad spectrum of xylan-degrading enzymes with different physicochemical properties (14,15), and most of which belongs to family G/11. The XynB xylanase with high activity was prepared from *A. niger* SCTCC 400264 in our previous study. We here report on the protein engineering of the XynB xylanase toward enhanced alkalophilic performances. We explored the strategy of changing pH-stability profiles by altering p*K*a values of catalytic residues and governing the ionization states of their side chains of its titrable amino acid residues through computational designed mutations.

MATERIALS AND METHODS

Bacterial hosts strains and plasmids The recombinant plasmid pMD18-XYNB with *A. niger* xynB gene produced previously was used as template for

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http://dx.doi.org/10.1016/j.jbiosc.2012.12.006

sitedirected-mutagenesis, *Escherichia coli* JM109 was used as a cloning host and DNA propagation and was grown in LB medium. *E. coli* BL21 was used to express recombinant protein using the expression vector pET32a (Novagen/Merck).

Computational design procedure To avoid modification of residues in the close proximity of the two catalytic glutamic acids, the targets were residues outside the active cleft. Modeling of the mutant was manipulated by the SWISS-MODEL server homology modeling pipeline (16,17). The crystallographic structure of wheat *Triticum aestivum* xylanase XIPI (18) was used. The web server called PROPKA is available for the calculation of protein p*K*a values (19). It can access files directly from the PDB. A detailed description of the PROPKA for p*K*a values in a protein has been published. Following the design runs, an output of proposed mutations resulting in altering p*K*a values of catalytic residues is generated.

Mutant construction A three step polymerase chain reaction (PCR) was used to construct the mutants of N151E and T108V. In the first step, two fragments with overlapping sequences were amplified using the respective primers. The overlapping regions were used for self-priming in the second step of the overlapping PCR. In the third step, the outer flanking primers were used to amplify the full-length mutant gene. The first PCR step amplification was done with 10 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 55°C, and 2 min extension at 72°C. The overlapping PCR step was done for 10 cycles of 1 min at 95°C, 1.5 min at 50°C and 2 min at 72°C. The third PCR step condition was similar to the first step except that the extension time was 4 min. In all the PCR steps, a final extension was carried out for 7 min at 72°C. The PCR was carried out using a Life Express PCR System TC96 (Bioer Technology, China) and a high fidelity PCR enzyme mix (Takara, Japan). The mutagenic primers used were: S108V forward, CACCGTTACCGTTGATGGA TCCGTTTAC; S108V reverse, GGATCCATCAACGGTAACGGTGCCCTT; N151E forward, ACCTCCGAACACTTCAATGCTTGGGCT; N151E reverse, GCCCAAGCATTGAAGTGTTCGG AGGTGG; Q177R forward, TCACAACTACCAGATCGTGGCTACC; Q177R reverse, GCTCC GGTAACCCTCGGTAGCCACGA; multiple forward, GGAATTCTCGACCCCGAGCTCGACC GGCGAGAA and multiple reverse, CCCCCGCCTTCATTAAAAGCTTCATTCACCAC.

Expression, proteins purification and analysis The *E. coli* strains were grown at 37°C in Luria-Bertani broth. Ampicillin (Sigma Chemical) was used at 100 μg/mL. After transformation by shuttle plasmids carrying the different mutations, recombinant XynB proteins were produced as follow: genetic engineering bacteria were induced at OD600 of 0.6–0.8 by the addition of 0.2 mM isopropylthion-β-D-galactoside (IPTG) and cultured for another 4 h at 30°C. Culture supernatants were pelleted by centrifugation at 5000 r/min for 10 min and resuspended in 1× phosphate buffer (PB). Cells were ruptured by ultrasonic. The lysate was clarified by centrifugation at 12,000 r/min for 10 min. The supernatant containing His-xylanase was purified by Ni^{2+}-NTA affinity chromatography. Protein expression and purification were assessed by standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie blue staining. Protein concentrations were determined by the method of Bradford (20). The fractions containing xylanolytic activity were collected. Solutions were concentrated and dialyzed against 1× phosphate buffer (PB).

pH-Dependence of xylanase activity Endo-β-1,4-xylanase (EC 3.2.1.8) activity was measured by the dinitrosalicylic acid method (DNS) (21). Aliquots (40 μL) of diluted enzyme were mixed with 360 μL of a 1% suspension of oat spelled xylan (Sigma Chemical Co.) at different pH and incubated for 10 min at 50°C. Buffers were 0.05 M HCl/KCl (pH 2.0), 0.05 M sodium citrate (pH 2.5–6.0), 0.05 M sodium phosphate (pH 6.0–7.0), 0.05 M Tris/HCl (pH 7.5–8.5) and 0.05 M Gly-NaOH(pH 9.0–10.0). One international unit (IU) is defined as the amount of enzyme that releases 1 μmole of reducing sugar per minute.

pH-Dependence of xylanase stability To test the functional pH stability of XynB and mutants, residual endoxylanase activity was measured after 2 h of incubation (50°C) of the enzymes in buffers with varying pH (pH 2–10). Following incubation, pH values were adjusted to pH 5.5 by diluting the enzyme solutions 10 times in sodium phosphate buffer (250 mM) and endoxylanase activities were determined using the DNS method as described above.

RESULTS

Rational design A novel computational approach was used to enhance alkalophilic performances of XynB xylanase. The strategy involved altering p*K*a values of catalytic residues and governing the ionization states of their side chains of its titrable amino acid residues through computational designed mutations.

For p*K*a calculation, the structure models of the wild-type xylanase and its mutants were generated by the fully automated homology-modeling pipeline SWISS-MODEL (Fig. 1). In the wild-type enzyme, mutant S108V and mutantN151E, the Asp 19 side chain does not interact with other residues. Modeling of the Q178R mutant is relatively straightforward, leading to the formation of two backbone hydrogen bonds of Asp19–Gly21 and Asp19–Asn 43, which induce a shortening of the Glu84–Glu175 distance from 6.02 Å to 5.92 Å. The replacement of Gln178 by Arg can give rise to several conformations of the latter side chain. All starting conformations lead to models in which Arg 177 is strongly bonded to Asp 19 and Tyr 177 by coulombic interaction. The formation of the salt bridge reorients the Arg119 side chain with respect to its position in the wild-type. The Arg119–Glu84 and Arg119–Glu175 distances change from 4.96 Å to 5.39 Å and from 9.88 Å to 7.82 Å, respectively. Moreover, a remarkable difference in the structures among the mutant Q178R, the wild-type enzyme, the mutant S108V and the mutant N151E was detected in Glu84. The Glu84 in Q178R is completely buried, and the Glu84 in the wild-type enzyme, the mutant S108V and the mutant N151E is 97% buried.

The p*K*a values of catalytic residues in the wild-type xylanase and its mutants were predicted by PROPKA 3.0. The p*K*a values of Glu84 and Glu175 in the wild-type xylanase are 6.60 and 7.88, respectively. The p*K*a value of Glu84 in S108V shifts down to 5.49, and Glu175 p*K*a value is not influenced. On the contrary, the p*K*a value of Glu84 in N151E still remains 6.60, whereas Glu175 p*K*a value shifts up to 7.89. However, the p*K*a shifts of Glu84 and Glu175 in Q178R are large. The p*K*a values of Glu84 and Glu175 in Q178R are 7.91 and 6.32, respectively (Table 1).

Expression and purification of recombinant xylanases The entire coding regions of wild-type xylanase and its mutants, were amplified by PCR respectively, and then were cloned into pET32a vector. They were expressed in different conditions in *E. coli* BL21(DE3). The recombinant xylanase variants were purified to near homogeneity on a Ni-NTA affinity column (Fig. 2). The specific activity of the wild-type enzyme was evaluated at 1021 ± 100 IU/mg at 50°C and its optimum pH 5.5, and under the same conditions the specific activity of the mutant enzymes of S108V, N151E, and Q178R were 968 ± 70 IU/mg, 1100 ± 100 IU/mg, and 1260 ± 100 IU/mg, respectively. With the exception of the S108V protein, which presented a slightly lower specific activity, all mutants showed increased specific activities at pH values of 5.5.

pH-Dependence of xylanase activity The activity of the four purified proteins, the wild-type XynB, S108V, N151E, and Q178R, was assayed at pH values between 2.0 and 10.0. The wild-type enzyme optimum pH was close to 5.5 (Fig. 3). Its activity was reduced by 80% at pH 2.0 and by 85% at pH 7.0, and was negligible at pH 8.0. The S108V mutant showed an activity profile similar to that of the wild-type enzyme. N151E was optimally active at pH 5.5. Moreover, it showed 25% of their maximum activities at pH 2.0 and 34% of their maximum activities at pH 7.0. But its activity was also negligible at pH 8.0 (Fig. 3). Interestingly, the mutant Q178R was optimally active at pH 6.0 and retained 37% of the maximum activity at pH 8.0.

pH-Dependence of xylanase stability The pH stability of the wild-type xylanase and its mutants was assessed after pre-incubation of the enzymes for 2 h at different pH values. As shown in Fig. 4, acid-induced inactivation of wt XynB was initiated once the pH dropped below pH 5, retaining 48% residual activity after pre-incubation at pH 4 under the experimental conditions, and only 21% residual activity after pre-incubation at pH 3. Alkaline-induced inactivation of wt XynB was initiated once the pH rose above pH 8.0, retaining 86% residual activity after pre-incubation at pH 9.0 under the experimental conditions and only retaining 34% residual activity after pre-incubation at pH 11. The S108V and N151E mutant showed a stability profile similar to that of the wild-type enzyme at pH 7.0–11.0, and showed more tolerance toward acid-mediated denaturation. On the contrary, the Q178R mutant showed a stability profile similar to that of the wild-type enzyme at pH 3.0–5.0, and showed more tolerance toward alkaline-mediated denaturation.