Site-directed Mutagenesis of a β-Glycoside Hydrolase from Lentinula edodes

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Received: 23 November 2018; Accepted: 23 December 2018; Published: 24 December 2018

Abstract: The β-glycoside hydrolases (LXYL-P1−1 and LXYL-P1−2) from Lentinula edodes (strain M95.33) can specifically hydrolyze 7-β-xylosyl-10-deacetyltaxol (XDT) to form 10-deacetyltaxol for the semi-synthesis of Taxol. Our previous study showed that both the I368T mutation in LXYL-P1−1 and the T368E mutation in LXYL-P1−2 could increase the enzyme activity, which prompted us to investigate the effect of the I368E mutation on LXYL-P1−1 activity. In this study, the β-xylosidase and β-glucosidase activities of LXYL-P1−1I368E were 1.5 and 2.2 times higher than those of LXYL-P1−1. Most importantly, combination of I368E and V91S exerted the cumulative effects on the improvement of the enzyme activities and catalytic efficiency. The β-xylosidase and β-glucosidase activities of the double mutant LXYL-P1−1V91S/I368E were 3.2 and 1.7-fold higher than those of LXYL-P1−1I368E. Similarly, the catalytic efficiency of LXYL-P1−1V91S/I368E on 7-β-xylosyl-10-deacetyltaxol was 1.8-fold higher than that of LXYL-P1−1I368E due to the dramatic increase in the substrate affinity. Molecular docking results suggest that the V91S and I368E mutation might positively promote the interaction between enzyme and substrate through altering the loop conformation near XDT and increasing the hydrogen bonds among Ser91, Trp301, and XDT. This study lays the foundation for exploring the relationship between the structure and function of the β-glycoside hydrolases.

Keywords: site-directed mutagenesis; β-xylosidase; β-glucosidase; catalytic efficiency; molecular docking

1. Introduction

As the biocatalysts, enzymes are widely used in the production of food products, commodities, and pharmaceutical intermediates [1,2]. The prompt developments in protein engineering technology have provided the useful tools for improving enzyme critical traits, such as stability and catalytic efficiency [3–8]. The common strategies for protein engineering include directed evolution and rational protein design [9,10]. Directed evolution is a method that mimics the natural evolution in the laboratory. It utilizes the error-prone PCR or DNA shuffling technique in combination with the high-throughput screening method to continuously accumulate the dominant mutations with improved characteristics of the enzyme [5,11–15]. Rational design is conducted based on the understanding of the catalytic mechanism or the enzyme structure in which the stereo-structure can sometimes be predicted by protein homology modeling technique [16–21]. The key amino acids that may affect the enzyme properties can be chosen for site-directed mutagenesis, which includes the single site-directed mutation, multiple site-directed mutations, and saturation mutation. For example, the thermostability of Geobacillus stearothermophilus xylanase was improved by directed evolution in combination with rational design and up to 13 amino acids were mutated during this process. The reaction temperature for maximum activity increased from 77 °C to 87 °C, and the catalytic efficiency increased by 90% [13].
Through DNA shuffling, site-directed mutation and saturated mutation, the stabilities and activities of the β-glucosidases from *Thermobifida fusca* and *Paebibacillus polymyxa* were significantly increased, making the enzymes more suitable for the bioconversion of cellulose [22]. By site-directed mutation of three His (His²⁷⁵, His²⁹₃, and His³¹₀) of the α-amylase in *Bacillus subtilis* into Asp, the catalytic efficiency of the mutant on the substrate was improved by 16.7 times compared with that of the wild type [23]. Additionally, the mutations of P140L/D416G significantly increased the catalytic efficiency of the mannanase from *Podospora anserina* [24]. All of the aforementioned examples suggested that protein engineering can promote the study of the enzyme structure-function relationship and can be used to design enzymes with improved or new functions, which will broaden the repertoire of enzymes.

The dual GH3 β-xylosidase/β-glucosidases, designated as LXYL-P1−1 and LXYL-P1−2, respectively, are enzymes of *Lentinula edodes* (strain M95.33) origin and have been cloned and characterized by our lab. The activity of LXYL-P1−2 is twice higher than that of LXYL-P1−1 [25]. In addition, both enzymes have been successfully expressed in *Pichia pastoris*. Moreover, both of them can specifically remove the xylosyl group from 7-xylosyl-10-deacetyltaxol (XDT) isolated from yew trees to produce 10-deacetyltaxol (DT) [25–27]. This product can be further acetylated into Taxol, a prominent anticancer drug originally isolated from Pacific yew tree [16,28–31]. In our previous study, the directed evolution of LXYL-P1−1 had been conducted. From the random mutant library created by error-prone PCR, we obtained a mutant LXYL-P1–2−EP2 (LXYL-P1–2T³⁶⁸E) that harbored the T368E mutation, which exhibited a 47% increase in its catalytic efficiency on XDT and elevated stability in the range of pH ≥ 6 compared with LXYL-P1–2 [32]. Recently, we found that the mutant LXYL-P1–1T³⁶⁸E also exhibited similar β-xylosidase and β-glucosidase activities compared with the high-active LXYL-P1–2, although the activities were lower than those of LXYL-P1–1A³⁷²T, LXYL-P1–1V⁹¹S (the most active single mutant), and the double mutant LXYL-P1–1A³⁷²T/V⁹¹S [33]. These results suggest that besides the putative active site residues [21] and in addition to positions 72 and 91, the amino acid in position 368 may also play an important role in terms of enzyme activity. In addition, we also observed that the double mutant LXYL-P1–1A³⁷²T/V⁹¹S even showed results 2.8- and 3-fold higher than the positive control LXYL-P1–2 on β-xylosidase and β-glucosidase activities, although the triple mutant LXYL-P1–1A³⁷²T/V⁹¹S/I³⁶⁸E did not exhibit increased activities compared with the same control [33]. These results prompted us to consider whether the I368E mutation in LXYL-P1–1 can also increase enzyme activities and whether the enzyme activities can be further improved by the combination mutation of V⁹¹S/I³⁶⁸E. In this study, the single mutant LXYL-P1–1I³⁶⁸E and the double mutant LXYL-P1–1V⁹¹S/I³⁶⁸E as well as their corresponding engineered yeasts were constructed, respectively. With respect to the results, we discovered that the mutant LXYL-P1–1I³⁶⁸E was indeed more active than LXYL-P1–1. Furthermore, the double mutant LXYL-P1–1V⁹¹S/I³⁶⁸E even surpassed the high-active LXYL-P1–2 in terms of the β-xylosidase and β-glucosidase activities. The possible mechanisms are further discussed.

2. Results and Discussion

2.1. The Volumetric and Biomass Enzyme Activities of the Recombinant Yeasts

In order to investigate the effect of I³⁶⁸E mutation on the enzyme activity, the recombinant yeast GS115-3.5K–P1–1I³⁶⁸E was constructed, and its volumetric and biomass enzyme activities were detected as described previously [32]. After induction by methanol for 4 days, the enzyme activities of GS115-3.5K–P1–1I³⁶⁸E had exceeded those of GS115-3.5K–P1–1 (Figure 1). At the induction time of 7 d, the volumetric and biomass β-xylosidase activities of GS115-3.5K–P1–1I³⁶⁸E reached 3.52 × 10⁶ U·L⁻¹ and 0.72 × 10⁵ U·g⁻¹, respectively, which increased by 21% and 18% compared with those of GS115-3.5K–P1–1 (2.92 × 10⁶ U·L⁻¹ and 0.61 × 10⁵ U·g⁻¹, respectively) (Figure 1a,b). Similarly, at the induction time of 7 days, the volumetric and the biomass β-glucosidase activities of GS115-3.5K–P1–1I³⁶⁸E arrived at 6.34 × 10⁶ U·L⁻¹ and 1.30 × 10⁵ U·g⁻¹, respectively, which increased by 23% and 21% compared with those of GS115-3.5K–P1–1 (5.14 × 10⁶ U·L⁻¹ and 1.07 × 10⁵ U·g⁻¹, respectively) (Figure 1c,d).
In our previous study, we found that the A72T mutation and V91S mutation exhibited a synergistic effect, in which the amino acid in position 91 of LXYL-P1−1 displayed a key role in affecting enzyme activity [33]. This synergistic effect was also observed in the double mutant V91S/I368E as shown in Figure 1. The enzyme activities of GS115-3.5K-P1−1V91S/I368E exceeded those of the control strain GS115-3.5K-P1−1I368E during the whole methanol induction period. Its volumetric and biomass β-xylosidase activities reached 11.51 × 10^6 U·L^{-1} and 2.32 × 10^5 U·g^{-1}, respectively, at the induction time of 7 days, which were 3.3- and 3.2-fold higher than those of GS115-3.5K-P1−1I368E (Figure 1a,b). Likewise, the volumetric and biomass β-glucosidase activities of GS115-3.5K-P1−1V91S/I368E reached 20.67 × 10^6 U·L^{-1} and 4.18 × 10^5 U·g^{-1}, respectively, at the induction time of 7 days, which were 3.3- and 3.2-fold higher than those of GS115-3.5K-P1−1I368E (Figure 1c,d).

Figure 1. Comparison of β-xylosidase and β-glucosidase activities between the recombinant yeasts GS115-3.5K-LXYL-P1−1, GS115-3.5K-LXYL-P1−1I368E, and GS115-3.5K-LXYL-P1−1V91S/I368E. The recombinant yeast GS115-3.5K-LXYL-P1−1 was used as the control. (a) Volumetric β-xylosidase activities. (b) Biomass β-xylosidase activities. (c) Volumetric β-glucosidase activities. (d) Biomass β-glucosidase activities.

2.2. Specific β-Xylosidase and β-Glucosidase Activities of the Mutants

The specific activities of the purified mutants were also detected. As shown in Figure 2, the β-xylosidase and β-glucosidase activities of LXYL-P1−1I368E reached 3.41 × 10^4 and 10.80 × 10^4 U/mg, respectively, which were 1.5 and 2.2 times as high as those of LXYL-P1−1 (2.33 × 10^4 and 4.93 × 10^4 U/mg, respectively), although the activities were lower than those of LXYL-P1−1E368T reported previously [33].
The β-xylosidase and β-glucosidase activities of the purified LXYL-P1−1V91S/I368E reached 11.04 × 10^4 and 18.27 × 10^4 U/mg, respectively, which were 4.7 and 3.7 times higher than those of LXYL-P1−1, and 3.2 and 1.7-fold higher than those of LXYL-P1−1I368E, and even 2.3- and 1.5-fold higher than those of LXYL-P1−2 (4.80 × 10^4 and 11.85 × 10^4 U/mg, respectively) (Figure 2). The results indicate that the I368E mutation in LXYL-P1−1 presented here has exhibited a positive effect on increasing the β-xylosidase and β-glucosidase activities. Meanwhile, the combination of V91S and I368E mutations had a synergistic effect on the increase of the β-xylosidase and β-glucosidase activities. Further, compared with the volumetric or biomass enzyme activity of the recombinant yeast represented in Figure 1, we found that the increased magnitude of the specific activity of LXYL-P1−1I368E was apparently higher than that of the volumetric or biomass activity of GS115-3.5K-LXYL-P1−1I368E. It means that the single mutation led to the decreased enzyme expression in the yeast host.

2.2. Specific β-Xylosidase and β-Glucosidase Activities of the Mutants

The kinetic parameters of LXYL-P1−1I368E and LXYL-P1−1V91S/I368E together with LXYL-P1−1I368T against XDT were detected and the data are summarized in Table 1. To LXYL-P1−1I368E, the increased affinity (K_m value: 0.35 vs. 0.50, mM) contributed to the slight improvement in its catalytic efficiency compared with that of LXYL-P1−1 (k_cat/K_m value: 4.46 vs. 4.10, s⁻¹⋅mM⁻¹). To LXYL-P1−1I368E, both the increased affinity (K_m value: 0.42 vs. 0.50, mM) and the increased turnover number (k_cat value: 2.41 vs. 2.05, s⁻¹) led to the significant increase in its catalytic efficiency compared with that of LXYL-P1−1 (k_cat/K_m value: 5.68 vs. 4.10, s⁻¹⋅mM⁻¹) (Table 1). Likewise, the significantly increased affinity (K_m value: 0.20 vs. 0.50, mM) and a similar turnover number (k_cat value: 2.06 vs. 2.05, s⁻¹) resulted in the 2.5-fold increase in the catalytic efficiency of LXYL-P1−1V91S/I368E compared with that of LXYL-P1−1 (k_cat/K_m value: 10.30 vs. 4.10, s⁻¹⋅mM⁻¹). In other words, the catalytic efficiency of LXYL-P1−1I368E against XDT was 1.3-fold higher than that of LXYL-P1−1I368T, and the catalytic efficiency of LXYL-P1−1V91S/I368E was nearly twice as high as that of LXYL-P1−1I368E (Table 1), and also surpassed that of LXYL-P1−1V91S (6.26 s⁻¹⋅mM⁻¹) [33].

2.3. Kinetic Analysis of the Mutated Enzymes against XDT

The kinetic parameters of LXYL-P1−1I368E and LXYL-P1−1V91S/I368E against XDT were detected and the data are summarized in Table 1. To LXYL-P1−1I368E, the increased affinity (K_m value: 0.35 vs. 0.50, mM) contributed to the slight improvement in its catalytic efficiency compared with that of LXYL-P1−1 (k_cat/K_m value: 4.46 vs. 4.10, s⁻¹⋅mM⁻¹). To LXYL-P1−1I368E, both the increased affinity (K_m value: 0.42 vs. 0.50, mM) and the increased turnover number (k_cat value: 2.41 vs. 2.05, s⁻¹) led to the significant increase in its catalytic efficiency compared with that of LXYL-P1−1 (k_cat/K_m value: 5.68 vs. 4.10, s⁻¹⋅mM⁻¹) (Table 1). Likewise, the significantly increased affinity (K_m value: 0.20 vs. 0.50, mM) and a similar turnover number (k_cat value: 2.06 vs. 2.05, s⁻¹) resulted in the 2.5-fold increase in the catalytic efficiency of LXYL-P1−1V91S/I368E compared with that of LXYL-P1−1 (k_cat/K_m value: 10.30 vs. 4.10, s⁻¹⋅mM⁻¹). In other words, the catalytic efficiency of LXYL-P1−1I368E against XDT was 1.3-fold higher than that of LXYL-P1−1I368T, and the catalytic efficiency of LXYL-P1−1V91S/I368E was nearly twice as high as that of LXYL-P1−1I368E (Table 1), and also surpassed that of LXYL-P1−1V91S (6.26 s⁻¹⋅mM⁻¹) [33].

### Table 1. Kinetic parameters for the mutated enzymes using XDT as the substrate.

| Enzyme     | v_max (µM·min⁻¹) | K_m (mM) | k_cat (s⁻¹) | k_cat/K_m (s⁻¹⋅mM⁻¹) |
|------------|-----------------|----------|-------------|----------------------|
| LXYL-P1−1  | 3.42 (±0.04)    | 0.50 (±0.01) | 2.05 (±0.02) | 4.10 **               |
| LXYL-P1−1I368T | 2.60 (±0.56) ** | 0.35 (±0.10) | 1.56 (±0.34) * | 4.46 *               |
| LXYL-P1−1I368E | 4.02 (±0.13) * | 0.42 (±0.01) * | 2.41 (±0.08) * | 5.74 ***              |
| LXYL-P1−1V91S/I368E | 3.43 (±0.01) | 0.20 (±0.01) ** ** | 2.06 (±0.01) | 10.30 ***             |

Note: Data are mean (±SD), n = 3, * p < 0.05 vs. LXYL-P1−1, ** p < 0.01 vs. LXYL-P1−1, *** p < 0.01 vs. LXYL-P1−1; ** p < 0.01 vs. LXYL-P1−1I368E, *** p < 0.001 vs. LXYL-P1−1I368E.
2.4. Substrate-Enzyme Molecular Docking

To further explore how these mutations affect enzyme activities, molecular docking between the mutants and the substrate XDT was conducted based on the virtual three-dimensional structure of LXYL-P1−1, which was previously predicted through molecular modeling homology [33]. As shown in Figure 3a,b, the 368th amino acid is located on the loop and at the surface of the predicted protein. The I368E mutation provided an opportunity to introduce geometrical alteration of the loop near the active pocket, which may lead to enhanced affinity to the substrate. In addition, the I368E substitution gave rise to a negative potential on the protein surface, which probably made the mutant more stable in such a micro-environment. As Ile368 is a nonpolar and hydrophobic amino acid and Glu368 is a polar and acidic amino acid, it is likely that the introduction of a polar residue in position 368 may contribute to enzyme stability, and had an important effect on improving enzyme activity. Moreover, the previous study suggests that the V91S might increase the hydrogen bonds among Ser91, Trp301, and XDT [33]. This phenomenon may also occur in the present study (Figure 3c,d), since the remarkably increased affinity of the double mutant LXYL-P1−1V91S/I368E (Km value: 0.12 mM) to the substrate XDT was observed (Table 1).

![Figure 3](image_url)

**Figure 3.** Partial view of enzyme-XDT docking. (a) Side view of LXYL-P1−1 with XDT, showing Val91 and Ile368. (b) Side view of LXYL-P1−1I368E with XDT, in which Ile368 are replaced by Glu368. (c) Side view of LXYL-P1−1V91S/I368E with XDT, in which Val91 and Ile368 are replaced by Ser91 and Glu368, respectively. (d) Enlarged view of molecular docking of LXYL-P1−1V91S/I368E with XDT, in which the increased hydrogen bonds among Ser91, Trp301, and XDT are indicated in red. The geometrical alteration of the loop near the active pocket is indicated in salmon. The carbon atoms of XDT are shown in orange. The nucleophile Asp300 (catalytic site), Val91/Ser91 and Ile368/Glu368 are colored in blue.
3. Materials and Methods

3.1. Plasmids and Strains

The recombinant plasmid pPIC3.5K-LXYL-P1−1 harboring the lxyl-p1−1 gene from *L. edodes* M95.33 was previously constructed in our laboratory. *Pichia pastoris* GS115-3.5K-P1−1 was constructed by transforming the plasmid pPIC3.5K-LXYL-P1−1 into the host strain *P. pastoris* GS115 (Mut*), and preserved at −80 °C prior to use [25].

3.2. Construction of the Recombinant Plasmids Expressed lxyl-p1−1I368E and lxyl-p1−1V91S/I368E

The lxyl-p1−1 variants harboring single site-directed mutation or double site-directed mutations were amplified using the PCR-based overlap extension method. The primers used for the amplification are listed in Table 2. For the construction of lxyl-p1−1I368E, the two individual fragments were amplified by Phusion DNA polymerase using primers P1−1-F/I368E-R and I368E-F/P1−1-R, respectively, with the plasmid pPIC3.5K-LXYL-P1−1 being used as a template. The PCR conditions for amplification consisted of 98 °C for 30 s, 30 cycles of 10 s at 98 °C, 30 s at 60 °C, 1 min at 72 °C, and a final 10 min extension at 72 °C. The PCR products were purified using a gel extraction kit (Transgen, Beijing, China). Later, the overlap extension was performed by mixing 100 ng of the two fragments in equimolar amounts with Phusion PCR buffer, dNTPs, and Phusion polymerase in a total volume of 25 µL. The PCR conditions for amplification were 98 °C for 30 s, 15 cycles of 10 s at 98 °C, 30 s at 60 °C, 72 °C for 30 s/kb, followed 10 min incubation at 72 °C. Then, 2 µL of the unpurified PCR product was further used as a template for the second round PCR. Additionally, P1−1-F and P1−1-R, Phusion PCR buffer, dNTPs, and Phusion polymerase were added into the PCR mixture in a final volume of 50 µL. The amplification was performed identically to the PCR reaction of the individual fragments. Finally, the fragment lxyl-p1−1I368E containing the I368E mutation was obtained. For the construction of lxyl-p1−1V91S/I368E, the plasmid pPIC3.5K-LXYL-P1−1 was also used as a template, and the three individual fragments were amplified using primers SP1−1-F/V91S-R, V91S-F/I368E-R, and I368E-F/P1−1-R, respectively. Next, the three independent fragments were fused by overlap extension PCR to gain lxyl-p1−1V91S/I368E. Finally, lxyl-p1−1I368E and lxyl-p1−1V91S/I368E were ligated into the expression vector pPIC3.5K at the *Bam*H I and *Not* I restriction sites to generate the expression plasmids pPIC3.5K-LXYL-P1−1I368E and pPIC3.5K-LXYL-P1−1V91S/I368E, respectively. The recombinant plasmids with site-directed mutations were confirmed by nucleotide sequence analysis.

### Table 2. The primers used for amplification of lxyl-p1−1 variants.

| Primer | Sequence (5’→3’) |
|--------|------------------|
| P1−1-F | CGCGGATCCATGTTCTCACAGCAAGAC |
| P1−1-R | TTTTCCTTTTGGCGCGCTGCAGTGGTGGTGG |
| V91S-F | GAATTAGCCAACATCACCTAGGGTTATAGGTGTTGTGCAGGATA |
| V91S-R | TACTCCCTGACAAACACCTATAAACCCTGAGGTGATGGCTAATT |
| I368E-F | CAAGATGAAAATCCACACACCACCTTTG |
| I368E-R | TGGATTTCTTTGACCGAGTTAATAG |

Note: The underlined is the restriction enzyme cleavage site. The mutated bases are indicated in box.

3.3. Construction of the Recombinant Yeast Expressed lxyl-p1−1I368E and lxyl-p1−1V91S/I368E

For construction of engineered *P. pastoris* strains containing multi-copy lxyl-p1−1I368E and lxyl-p1−1V91S/I368E, 10 µg of recombinant vectors (pPIC3.5K-LXYL-P1−1I368E and pPIC3.5K-LXYL-P1−1V91S/I368E) were linearized with *Sac* I and introduced into *P. pastoris* GS115 via electroporation transformation according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). The transformants were initially selected on MD plates (13.4 g/L yeast nitrogen base, 0.4 mg/L biotin, 20 g/L dextrose, and 15 g/L agar) and then screened for multiple integrants on YPD plates.
(10 g/L yeast extract, 20 g/L tryptone, 20 g/L D-glucose, and 15 g/L agar) containing 4 mg/mL G418. Genomic DNA of the transformants was extracted via TIANamp Yeast DNA Kit following the manufacturer’s instruction, and used for the further PCR analysis.

3.4. Induction Protein Expression of LXYL-P1−1I368E and LXYL-P1−1V91S/I368E

The recombinant yeasts harboring the lxyl-p1−1I368E and lxyl-p1−1V91S/I368E were firstly inoculated in 500-mL shake flasks containing 100 mL buffered minimal glycerol complex medium (BMGY) medium (containing 10 g/L yeast extract, 20 g/L tryptone, 13.4 g/L YNB, 0.4 mg/L biotin, 10 g/L glycerol, 100 mmol·L−1 potassium phosphate buffer, pH 6.0) at 30 °C with shaking at 200 rpm for 48–60 h. Then methanol was added every day to maintain 1% (v/v) for the induction of the gene expression.

3.5. Volumetric and Biomass Enzyme Activities Measurement of the Recombinant Yeasts

At the methanol induction stage, the volumetric and biomass β-xylanosidase and β-glucosidase activities of the recombinant yeasts were measured every day. The culture was harvested via centrifugation and was washed twice with dH2O, and the cell pellet was resuspended with dH2O in the same volume of the culture broth. Next, 10 µL of the cell suspension was added to 50 µL of 5 mmol·L−1 PNP-Xyl or PNP-Glu, and incubated for 20 min at 50 °C for the catalytic activity analysis. The volumetric and biomass β-xylanosidase and β-glucosidase activities were then evaluated as described previously [32].

3.6. Enzyme Activities Measurement of LXYL-P1−1I368E and LXYL-P1−1V91S/I368E

After 7 days of induction, the recombinant mutants were isolated and purified according to the method described in our previous report [25,32]. The β-xylanosidase and β-glucosidase activities of mutants were measured by detecting the amount of p-nitrophenol released from the substrate PNP-Xyl or PNP-Glu under the optimum reaction conditions. Next, 60 µL reaction volume contained 50 µL of 5 mmol·L−1 PNP-Xyl/PNP-Glc and 10 µL of diluted enzyme in 50 mmol·L−1 sodium acetate buffer with pH 5.0. The reaction was performed under 50 °C for 20 min. Reactions were terminated by adding 1 mL saturated Na2B4O7 solution. The enzymatic activity was assayed using spectrophotometry based on the absorbance at 405 nm. One unit of activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol·L−1 p-nitrophenol per minute.

3.7. Kinetic Study of LXYL-P1−1I368E and LXYL-P1−1V91S/I368E

The kinetic parameters of LXYL-P1−1I368E and LXYL-P1−1V91S/I368E against XDT were determined at the XDT concentration ranging from 0.039–5.0 mmol·L−1 as described previously [32]. DT formation was analyzed through HPLC. The kinetic data on XDT were processed by a proportional weighted fit using a nonlinear regression analysis program based on Michaelis–Menten enzyme kinetics. All data were presented as means ± SD of three independent repeats.

4. Conclusions and Perspective

In conclusion, the site-directed mutagenesis of the amino acid in position 368 of LXYL-P1−1 was conducted, and the mutant with the I368E mutation had exhibited increased β-xylanosidase and β-glucosidase activities. Moreover, combination of I368E and V91S could further significantly improve the enzyme activity and catalytic efficiency. The increased catalytic efficiency of LXYL-P1−1V91S/I368E on XDT was mainly due to the dramatic increase in the substrate affinity. Molecular docking analysis between the mutants and XDT deduced the possible molecular mechanism for the improved enzyme activities. Our results suggest that combination of two or more beneficial mutations should probably improve the enzyme activities. In the future, the saturation mutation on the 368th site of LXYL-P1−1 followed by the other combinatorial mutations (including A72T/I368E, A72T/I368T and V91S/I368T)
may be conducted to find more active mutants. The corresponding high-active mutant can be further used for the bioconversion of XDT to DT for the semi-synthesis of Taxol. This study provides the theoretical basis for the identification of the important key amino acid residues out of active sites that positively affect the activities of the $\beta$-glycoside hydrolases, and lays the foundation for further exploring the relationship between the structure and function of the $\beta$-glycoside hydrolases.

**Author Contributions:** J.-J.C. designed and performed the experiments and drafted the manuscript. X.L. helped to prepare the mutant proteins and perform kinetics measurement. T.-J.C. and J.-L.Y. help to analyze the data and revised the manuscript. P.Z. conceived and supervised the study and revised the manuscript.

**Funding:** This work was supported by the National Natural Science Foundation of China (Grant no 81573325), the National Mega-project for Innovative Drugs (Grant no 2018ZX0911001-006-001), the Fundamental Research Funds for the Central Universities (Grant no. 2017PT35001), and CAMS Innovation Fund for Medical Sciences (Grant no. CIFMS-2017-22M-2-004).

**Conflicts of Interest:** The authors declare no conflict of interest.

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**Sample Availability:** Sample of the compound, 10-deacetyltaxol, is available from the authors.