Structures of β-glycosidase LXYL-P1-2 reveals the product binding state of GH3 family and a specific pocket for Taxol recognition

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LXYL-P1-2 is one of the few xylosidases that efficiently catalyze the reaction from 7-β-xylosyl-10-deacetyltaxol (XDT) to 10-deacetyltaxol (DT), and is a potential enzyme used in Taxol industrial production. Here we report the crystal structure of LXYL-P1-2 and its XDT binding complex. These structures reveal an enzyme/product complex with the sugar conformation different from the enzyme/substrate complex reported previously in GH3 enzymes, even in the whole glycohydrolases family. In addition, the DT binding pocket is identified as the structural basis for the substrate specificity. Further structure analysis reveals common features in LXYL-P1-2 and Taxol binding protein tubulin, which might provide useful information for designing new Taxol carrier proteins for drug delivery.
Taxol (generic name: paclitaxel), a rare natural product mainly generated by yew bark, is the well-known blockbuster anticancer drug. It promotes tubulin assembly into microtubules and prevents their disassembly. However, the natural level of Taxol is extremely low, while the content of 7-β-xylosyl-10-deacetyltaxol (XDT) can be up to 25 times of Taxol. XDT is often regarded as the waste during Taxol extraction process, causing both resource loss and potential environmental pollution. Compared with Taxol, XDT lacks the C10 hydroxyl group but harbors an additional β-xylosyl group at the C7 position. If the xylosyl group is removed, the resultant 10-deacetyltaxol (DT) can be used as a precursor for Taxol preparation. Therefore, the β-xylosidase catalyzing the removal of xylosyl from XDT would play a prominent role in reducing both of the resource waste and pollution to environment. However, a lot of commercially available β-xylosidases have been demonstrated to possess no activity on releasing the xylosyl residue from XDT. Recently, we identified two bifunctional β-xylosidase/glucosidase (named as LXYL-P1-1 and LXYL-P1-2) from Lentinula edodes, which belong to GH3 family and can efficiently convert XDT into DT. By combining LXYL-P1-2 and an engineered acetyltransferase, we have constructed an in vitro one-pot reaction system for converting XDT into Taxol.

The catalytic specificity and higher efficiency of LXYL-P1-2 prompted further investigation of its structure-function relationship. Here we present the crystal structures of LXYL-P1-2 and its complex with XDT. The binding mode of xylose group sheds light on the catalytic mechanism for GH3 enzymes. DT binding pocket elucidates the structural basis of substrate specificity. Structural comparison of LXYL-P1-2 and tubulin suggests a possible common feature for designing Taxol binding protein.

### Results

**Structures of LXYL-P1-2 in substrate free form.** Highly glycosylated proteins, such as LXYL-P1-2, are greatly difficult to be crystallized. To improve formation of good quality crystals, LXYL-P1-2 was endoglycosidase-treated before crystallization. The
crystallographic statistics for data collection and structure refinement are summarized in Table 1. The first 43 residues were missing in the electron density map, partially supporting the existence of the KEX2 cleavage site (Ile–Phe–Arg–Arg33). The first amino acid of the mature protein was then verified to be Asp36 by N-terminal sequencing.

LXYL-P1-2 exists as a 222 symmetric tetramer in the asymmetric unit (Fig. 1b) (PDB code 6JBS), consistent with the molecular weight measured by Gel filtration experiment. The structure of the four monomers are essentially same without any remarkable difference. Each monomer comprises three domains as some GH3 members. Domain 1 folds into a TIM barrel-like structure and contains the residues from Asp36 to Gly365. Domain 2 (residue 398–600) is an α/β sandwich, in which five parallel β-strands and one antiparallel β-strand are sandwiched by five α-helices. Domain 1 and domain 2 is connected by linker 1 (residue 366–397). Domain 3 (residue 664–803) is connected to domain 2 by linker 2 (residue 601–663) and has a fibronectin type III (FnIII) fold. Seven Asn residues (81, 272, 342, 385, 457, 576, and 635) are found to link with different types of oligosaccharide even after treated by endoglucosidase H (Table 2).

Structure of E529Q mutant co-crystallized with XDT. To obtain the substrate binding structure, mutant E529Q was co-crystallized with XDT (PDB code 6KJ0). There are two monomers in the asymmetric unit. The electron density clearly shows the existence of xylose and DT (Fig. 2a). The xylose adopts a pyranose configuration. It was surprised to find that the glycosidic bond between xylose and DT was broken in both of the monomers. Although the E529Q mutant did not show catalytic activity in the standard enzymatic assay, it may still have weak activity to digest the glycosidic bond of XDT during the crystallization time of one week. Thus, this structure should be regarded as the enzyme/product complex.

Sequence alignment of LXYL-P1-2 indicates that Asp300 and Glu529 might act as the catalytic nucleophile and the acid/base residues, respectively. In LXYL-P1-2 structure, the distance of these two residues is 5.5 Å, consistent with the proposed retaining catalytic mechanism in GH3 family. In the substrate free enzyme, Glu529 forms hydrogen bond to the side-chain of Arg218 and the catalytic mechanism in GH3 family. In the substrate free enzyme, Glu529 forms hydrogen bond to the side-chain of Arg218 and the side-chains of Asp109, Arg174, Lys207, His208, and Asp300 stabilize the glycosylation does not contribute to the enzymatic activity.

Site-directed mutations of the XDT binding region. According to the enzyme-substrate complex structure, a number of residues at xylose and DT binding site were subjected to site-directed mutations to inspect their influence on the enzymatic activity. Alanine scanning mutations were carried out and the activities of the mutants are summarized in Fig. 2e. Unsurprisingly, the mutations of conserved catalytic residues all showed an inactive or very low active. Decased activities were observed on F324A, L328A, and L325A mutants, which locate at the recognition area for benzoate group of DT. Mutation S228A destroyed the potential hydrogen bond between OG-Ser228 and N-Tyr221 (3.0 Å in E529Q), making it easier for loop Ile–Gln292 to move. Mutation S449A might provide more hydrophobicity in DT binding pocket. It is surprised that mutation S91A, which hydrogen bonds to Asp109, increased the enzyme activity, too.

**Table 1** Statistics of data-collection and refinement.

| Data sets                  | Native(6JBS) | E529Q-XDT(6KJ0) |
|----------------------------|--------------|------------------|
| Diffraction data           | P43222       | C222             |
| Space group                |              |                  |
| a, b, c (Å)                | 131,139,139,385.9 | 79.9,182,224.15  |
| α, β, γ ()                 | 90, 90, 90   | 90, 90, 90       |
| Resolution range (Å)       | 50.2 (4.29–4.20) | 50.2–227 (2.30–2.27) |
| Number of unique reflections | 133487       | 76271            |
| Data completeness (%)      | 99.9 (99.1)  | 93.5 (94.1)      |
| Redundancy                 | 12.8 (7.1)   | 8.6 (8.4)        |
| ⟨Ri/σi⟩                   | 23.6 (2.9)   | 27.1 (4.0)       |
| Rmerge (%)                 | 0.155 (0.667) | 0.122 (0.548)    |
| R-factor/Refinement        | 0.184/0.251  | 0.192/0.251      |
| Number of reflections used | 126287       | 72397            |
| r.m.s.d. bond length (Å)   | 0.009        | 0.017            |
| r.m.s.d. bond angles (°)   | 1.527        | 2.040            |
| Mean B factor (Å²)         |              |                  |
| Protein main-chain atoms   | 33.9         | 33.8             |
| Protein side-chain atoms   | 35.4         | 34.5             |
| Water molecules            | 32.9         | 33.8             |
| Glycan                     | 51.8         | 55.6             |
| Tris                       | 42.1         | -                |
| DT                         | -            | 55.1             |
| Xylose                     | -            | 34.8             |
| No. of atoms               |              |                  |
| Protein                    | 22984        | 11433            |
| Water molecules            | 1544         | 683              |
| Glycan                     | 952          | 493              |
| Tris                       | 32           | -                |
| DT                         | -            | 118              |
| Xylose                     | -            | 20               |

The free R factor was calculated using 5% of reflections omitted from the refinement

The r.m.s.d. bond angles (°) 1.527 2.040

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| Protein main-chain atoms   | 33.9         | 33.8             |
| Protein side-chain atoms   | 35.4         | 34.5             |
| Water molecules            | 32.9         | 33.8             |
| Glycan                     | 51.8         | 55.6             |
| Tris                       | 42.1         | -                |
| DT                         | -            | 55.1             |
| Xylose                     | -            | 34.8             |

The free R factor was calculated using 5% of reflections omitted from the refinement

The r.m.s.d. bond angles (°) 1.527 2.040

**Table 2** Glycan modification of LXYL-P1-2.

| Residue | Glycan structure |
|---------|------------------|
| Asn81   | GlicNac-β1-4-GlicNac-α1-4-Man |
| Asn277  | GlicNac-β1-4-GlicNac-β1-4-Man-α1-4-Man |
| Asn342  | GlicNac |
| Asn385  | GlicNac |
| Asn457  | GlicNac-β1-4-GlicNac-α1-4-Man-α1-3-Man-β1-6-Man-α1-2-Man-α1-2-Man |
| Asn576  | GlicNac |
| Asn635  | GlicNac |

indicates that the glycosylation does not contribute to the enzymatic activity.
This might indicate that Asp109 that interacts with xylose needs more flexibility during catalysis.

**Discussion**

Comparison with other GH3 enzymes shows that the crystal structures of LXYL-P1-2 provide the detailed information of xylosidase with the activity to hydrolyze 7-β-xylosyl-10-deacetyltaxol for the first time to our knowledge. Other structures similar to LXYL-P1-2 were found by using Dali server. As expected, the overall fold of LXYL-P1-2 is resembled to some three-domain GH3 members. The RMSD of main-chain atoms is 1.3 Å when superimposing LXYL-P1-2 to the most similar structure TnBgl3B. In the structure of LXYL-P1-2, eight cis-peptide bonds are found in Asp109-Gly110, Ala159-Pro160, Gly164-Pro165, Lys207-His208, Trp209-Ile210, Met319-Pro320, Ala381-Pro382, and Leu419-Pro420. Six of these cis-peptide bonds are conserved in many GH3 members, except for Ala381-Pro382 that makes a sharp turn involved in DT binding pocket. In addition, two intra-molecular disulfide bonds are formed by Cys266-Cys277 and Cys441-Cys451. These disulfide bonds are also conserved in most of GH3 members. Structure comparison also shows that the conformations of the residues around the sugar binding site, including the catalytic nucleophile Asp300 and the acid/base Glu529 are strictly conserved (Fig. 2d). This suggests LXYL-P1-2 share the common hydrolytic mechanism in GH3 family.

Interestingly, the xylose ring orientation rotated by about 60 degrees compared to the glucoses found in other GH3 enzyme structures as in HjCel3A. As shown in Fig. 2d, the OH-1,-2,-3 groups of xylose is corresponded to the OH-2,-3,-4 of glucose. The OH-1 of glucose points outwards of the active site, where the +1 group of substrate could be linked. The glucose soaked in crystals mimics the substrate binding state. In LXYL-P1-2, the xylose is generated from the hydrolysis of XDT and represent the product binding state, in which the OH-1 group is away from DT, with the distance between C1 of xylose and O7 of DT being 4.3 Å. If the xylose is orientated as the in HjCel3, the distance between C1 and O7 would be only 3.0 Å, indicating a good position to form the glycosidic bond. Therefore, the glucose soaked in HjCel3 crystals might mimic the substrate binding state. In LXYL-P1-2, the xylose is generated from the hydrolysis of XDT and might represent the product binding state. Both in the substrate and the product states, although the C1 atom of xylose in LXYL-p1-2 and the C1 atom of glucose in HjCel3 are at different positions, both of them are close to the nucleophile residue (Asp300 in LXYL-P1-2 and Asp236 in HjCel3, with the C1-OD1 distances of 2.7 Å and 2.8 Å).

In the proposed retaining mechanism of glycosidase, a covalent intermediate complex is expected to be formed by the sugar group of substrate and the nucleophile residue. The structures of xylose and glucose described above might depict the two states before and after the covalent intermediate stage. This might be the first report elucidating the rotation of the sugar ring from substrate binding to product forming states.

Besides comparison above, the tetramer formation attracts our attention. The catalytic pocket of each monomer faces to the outside of tetramer. The tetramer formation seems not in influence on the active pocket, but contributes to the highly thermal...
stability of LXYL-P1-2. Two interfaces are defined in the LXYL-P1-2 tetramer. On interface A, His118, Tyr390, Asn392, Arg394, Arg426, Tyr436, Glu479, Gln482, Asn489, and Glu491 form a number of hydrogen bonds between the two monomers, while Y473-I486*, L478-Y436*, L456-V438* (*indicates the residue from the neighboring monomer) form hydrophobic pairs. On interface B, Arg678-Tyr363*, Asp675-Lys66* form hydrogen bonds. In addition, Van der Waals interactions of Ser62-Trp679*, Thr368-Phe710*, and Asn369-Val757 also contribute to interface too. The interface A seems conserved as in AaBGL (Fig. 1b). However, the modified oligosaccharides are involved in dimer formation11. In LXYL-P1-2, there is no glycan modification close to this interface. The interface B was never reported in GH3 family. KmrBgl12 and AoβG13 were reported to be a tetramer, but their dimer-dimer interfaces are different from that in LXYL-P1-2.

Compared to other GH3 enzymes, the sequence and structural variations mainly come from the loops, especially those forming the substrate binding pocket (Fig. 3). Loops of residues 220–232, 324–328, 379–383, 446–450, and 529–530 form the DT binding pocket. As shown in the sequence alignment, the first four loops are conserved in LXYL-P1-1 and LXYL-P1-2 but are quite different from other GH3 enzymes. In fact, when superimposing

Fig. 3 Sequence alignment. Sequence alignment was done on the online sever of T-coffee23 and ESPript sever was used for result display24. Cysteine in the protein are shown by green figure, and the DT specific residues are shown by red points. XDT binding and catalytic domain are highlight in yellow.
other GH3 enzymes structures with LXYL-P1-2, they may have residues occupy the position of DT binding, such as Val298 and Phe225 in TnBgl3B, Trp57, Phe260, and Trp43 in HjCel3A, Trp224, Leu286, and Tyr310 in KmBgl12. The DT specificity results from the loop sequences, with the residues contributing hydrophobic side-chains towards the binding DT molecule. This suggests the pocket size, shape and hydrophobic environment are critical to DT recognition. As shown in Fig. 2e, the mutants with large hydrophobic side-chain removed decrease the enzymatic activity. In contrast, mutations of the surrounding residues S91A and S449A, which increase the hydrophobicity, could improve the catalytic ability.

To confirm the importance of the DT-binding loops, we purified the recombinant TnBgl3B that shares the same sugar binding site but different DT-binding loops, and tested its activity on PNP-Glc, PNP-Xyl, and XDT, respectively. The results showed that although TnBgl3B exhibited considerable activities on PNP-Glc and PNP-Xyl, the activity on XDT was undetectable (Table 3). In order to find more candidate enzymes with XDT-xidosidase activity, we searched the whole genome in EXPASY (Table 3). In order to find more candidate enzymes with XDT-xidosidase activity, we searched the whole genome in EXPASY blast server [https://web.expasy.org/blast/]. No protein except LXYL-P1-1 and LXYL-P1-2, are found with the similar DT-binding proteins.

Further structural analysis indicates that Ile222, Ile224, Ile322, Val227, and Gln229 in LXYL-P1-2 have the similar spatial distribution as Leu217, Leu219, Leu230, Leu275, and Asp282 in tubulin (Fig. 4c). It is interesting that the three benzene rings of DT and Taxol are also in the same spatial position when superposed (Fig. 4c), which partially supports the conserved spatial distribution of binding pockets in Taxol binding. Besides the structural information, the results of our enzyme catalytic experiments demonstrate that Phe224 and Leu234 in the benzoylamino region is critical for substrate recognition (Fig. 2d).

Table 3 Specific activity of TnBgl3B and LXYL-P1-2 against PNP-Xyl, PNP-Glc and XDT.

|        | PNP-Xyl (U/mg × 10^4) | PNP-Glc (U/mg × 10^4) | XDT (U/mg × 10^4) |
|--------|-----------------------|-----------------------|------------------|
| LXYL-P1-2 | 7.19 ± 0.87           | 15.59 ± 2.09          | 3.27 ± 0.24      |
| TnBgl3B  | 1.60 ± 0.03***        | 11.86 ± 0.70***       | ND               |

Values represent mean ± s.d. of triplicates from a representative experiment (n = 3 experiment replicates).

Methods

Materials and strains. The plasmid pPIC3.5K-lytl-p1-2 was cloned in our lab5, 16. The TnBgl3B gene (GenBank: ABL29899.1) was synthesized by SynBio Research Platform at Tianjin University (Tianjin, China). Plusion polymerase, restriction enzymes, and T4 ligase were purchased from New England Biolabs (Ipswich, MA).

Construction of active-site mutants of LXYL-P1-2 and TnBgl3B recombinant strain. The plasmid pPIC3.5K-lysl-p1-2 was used as DNA template. The L-alanine scanning mutations and other active-site mutations were all obtained by means of the site-directed mutagenesis technique with Phusion High-Fidelity DNA Polymerase (NEB) by using whole-plasmid amplification PCR. All products were sequenced to ensure that no base change other than designed. The plasmids were extracted, linearized with SacI and transformed into the Pichia pastoris GS115 competent cells for expression.

Protein expression, purification and activity assay. The heterologous expression and deglycosylation of LXYL-P1-2, as well as the mutants were same to the pre-vious article5, 6. E. coli cells with pET-28a-TnBgl3B recombinant plasmid were grown overnight at 37 °C and 200 r.p.m. in 10 ml Luria-Bertani (LB) medium containing kanamycin (50 μg/ml) in a shaking flask. The overnight culture was subcultured in 100 ml fresh LB medium at final concentration of 1% (OD), and grown at 37 °C and 200 r.p.m. for 2–3 h until OD600 reached 0.8. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM and the cell culture was incubated for an additional 20 h at 24 °C and 200 r.p.m. The protein was purified by Ni Sepharose 6 Fast flow resin and Agilent ZORBAX GF-450 gel-filtration column. The enzyme activities of LXYL-P1-2 were tested as reported previously5.

Crystallization, data collection, and structure determination. LXYL-P1-2 protein was concentrated to 10 mg/ml for crystallization. Both native crystal and complex crystal were grown at 16 °C with the hanging drop vapor diffusion.
method. For complex crystallization, E529Q protein was mixed with XDT before setting up. Native crystal and complex crystal grew in the solution contain 13% PEG3350, 0.1 M Tris-HCl pH8.5, 0.2% MgCl2 and 15% PEG3350, 0.1 M Hepes pH 7.5, 0.2% MgCl2, respectively. Diffraction data were collected in BL17U of the Shanghai Synchrotron Radiation Facility (SSRF). Data were processed using HKL2000. The native crystal diffracted to 2.4 Å (PDB code 6JBS), while the E529Q-XDT complex crystal diffracted to 2.27 Å (PDB code 6KJ0).

The native structure was solved by Phaser in CCP4 suit with TnBgl3B structure (PDB code 2 × 40) as the searching model. The complex structure was solved using native structure as the serching model. Refmac5 was used for strucure refinement. Coot was used for model building. All the structure figures were prepared using PyMol (https://pymol.org).

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
The coordinates of the crystal structures are deposited in Protein Data bank with the entrance codes of 6JBS and 6KJ0.

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Author contributions
L.Y. conducted the X-ray experiments and analyzed the experimental data. T.C., F.W., L.L., W.Y., Y.S., J.C. and W.L. prepared the protein samples and finished the activity assay. W.G. P.Z., L.Y. and T. C. wrote the paper.

Competing interests
The authors declare no competing interests.

Additional information
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