Comparison of glycoside hydrolase family 3 \(\beta\)-xylosidases from basidiomycetes and ascomycetes reveals evolutionarily distinct xylan degradation systems

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Xylan is the most common hemicellulose in plant cell walls, though the structure of xylan polymers differs between plant species. Here, to gain a better understanding of fungal xylan degradation systems, which can enhance enzymatic saccharification of plant cell walls in industrial processes, we conducted a comparative study of two glycoside hydrolase family 3 (GH3) \(\beta\)-xylosidases (Bxls), one from the basidiomycete Phanerochaete chrysosporium (PcBxl3), and the other from the ascomycete Trichoderma reesei (TrXyl3A). A comparison of the crystal structures of the two enzymes, both with saccharide bound at the catalytic center, provided insight into the basis of substrate binding at each subsite. PcBxl3 has a substrate-binding pocket at subsite -1, while TrXyl3A has an extra loop that contains additional binding subsites. Furthermore, kinetic experiments revealed that PcBxl3 degraded xylooligosaccharides faster than TrXyl3A, while the \(K_M\) values of TrXyl3A were lower than those of PcBxl3. The relationship between substrate specificity and degree of polymerization of substrates suggested that PcBxl3 preferentially degrades xylobiose (X2), while TrXyl3A degrades longer xylooligosaccharides. Moreover, docking simulation supported the existence of extended positive subsites of TrXyl3A in the extra loop located at the N-terminus of the protein. Finally, phylogenetic analysis suggests that wood-degrading basidiomycetes use Bxls such as PcBxl3 that act efficiently on xylan structures from woody plants, whereas molds use instead Bxls that efficiently degrade xylan from grass. Our results provide added insights into fungal efficient xylan degradation systems.

Woody and herbaceous biomasses are sustainable sources of biofuel and bio-based chemicals that can replace fossil resources, and enzymatic saccharification is a mild and effective way to utilize them. In nature, fungi degrade polysaccharides such as cellulose and hemicellulose, which account for about 70% of plant cell walls, with producing a variety of enzymes to support their growth. Therefore, the characterization of these enzymes can provide an insight into not only fungal degradation system, but also efficient enzymatic saccharification of the cellulosic biomass. Since some hemicelluloses are present on cellulose crystals, degradation of hemicellulose is crucial. Xylan is a common hemicellulose in the secondary cell wall of Angiosperms (1). The main chain of xylan is composed of \(\beta\)-1,4-linked xylose residues, and the degree of polymerization (DP) is about 80 to 150, regardless of the species and tissue. In contrast, the polysaccharides decorating the main chain differ greatly among plant species. Fungi produce various enzymes to mediate degradation of xylan into xylose (2). These enzymes are classified into two types, of which one degrades the main chain and the other degrades the side chains (3). The xylan main chain is degraded into xylooligosaccharides with low DPs by xylanase, and these products are further degraded into xylose by \(\beta\)-xylosidases (Bxls). In contrast to this simple degradation system, the digestion of polysaccharides decorating the main chain differs depending on their nature. Many kinds of enzymes are involved in cleaving polysaccharides attached to the main chain, including arabinofuranosidases, glucuronidases, and acetyl esterases.

Bxls are key enzymes for fungi to utilize xylan, because, unlike xylanase, they efficiently produce the monosaccharide xylose. Although Bxl works at the end of the xylan degradation system, it has to degrade a variety of substrates because xylanases produce diverse types of small xylooligosaccharides with various DPs and side chains, depending on the type of target polysaccharide (4).

Glycoside hydrolases (GH) family 3 (GH3) is one of the largest families in the Carbohydrate-Active enZymes (CAZy) database http://www.cazy.org/ (5) and includes enzymes such as \(\beta\)-glucosidase (Bgl), N-acetylhexosaminidase, oligoxylglucan \(\beta\)-glycosidase, and \(\beta\)-xylosidase (Bxl). All of them are exo-acting enzymes, which cleave one residue at a time from the nonreducing end of oligo- or polysaccharides, and their substrate specificity is determined by the architecture at subsite -1. It has also been reported that the DP of the substrate is important for substrate specificity. In kinetic studies with the Phanerochaete chrysosporium GH3 enzyme Bgl3A, for example, longer laminarioligosaccharides are better substrates.

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In the case of the GH3 Bgl from *Kluyveromyces marxianus* (*Km*BglI), the PA14 domain supports binding of longer substrates (7). But, in contrast to BglIs, little is known about either the substrate specificities of Bxls toward various xylooligosaccharides or the Bxl subsite structures.

The GH3 Bxl from the ascomycete *Hypocrea jecorina* (anamorph, *Trichoderma reesei*) (8), TrXyl3A, is one of the best-studied GH3 enzymes as regards substrate specificity, especially its activity toward substituted xylooligosaccharides (9). *T. reesei* has an arsenal of enzymes well suited for the degradation of grass biomass (10). For instance, *T. reesei* has several xylanases that belong to GH families 10, 11, and 30 (11–13). However, it has only one Bxl, named TrXyl3A, which belongs to GH3. TrXyl3A can degrade some substituted xylooligosaccharides, such as 2,3-α-D-glucuronyl-xylotetraose (XUXX) and 2,3-α-L-arabinofuranosyl-xylotetraose (XA2XX) (14). In contrast to ascomycetes fungi, there is limited knowledge about xylan degradation by basidiomycetes. *P. chrysosporium* is one of the best-studied white-rot fungi (15). It produces several xylanases belonging to GH families 10 (GH10) and 11 (GH11) (16), as well as a GH3 Bxl, PcBxl3, and degrades hardwood. The xylan-degrading enzymes of *P. chrysosporium* have not yet been well characterized.

Here, we describe structural and kinetic comparisons of two β-xylosidases from *P. chrysosporium* and *T. reesei*. The ligand-bound structures of these two enzymes account well for the kinetic differences, which are consistent with the evolution of the different xylan-degrading machineries required to degrade the preferred growth substrates of basidiomycetes and ascomycetes.

### Results

#### Sequence analysis

PcBxl3 and TrXyl3A contain 743 and 777 amino acid residues, respectively. Since the sequence identity between the two enzymes is 54%, the distributions of secondary structures in the two GH3 enzymes are quite similar, as shown in Figure 1. The most significant difference between the two GH3 β-xylosidase structures is an α-helix located at the N-terminal region of TrXyl3A. This additional N-terminal region containing the α-helix of TrXyl3A consists of 27 amino acids. There are also ten extra amino acids at the C-terminal region of TrXyl3A, but no secondary structure is predicted for them.

#### Overall structures of PcBxl3 and TrXyl3A

To uncover the structural differences between PcBxl3 and TrXyl3A, the crystal structures of both enzymes were solved. The crystal structure of TrXyl3A bound with thioxylobiose was determined by molecular replacement (MR) using free TrXyl3A (Protein Data Bank (PDB) ID: 5A7M, chain B) as a search model, and the crystal structure of free PcBxl3 was determined by MR using TrXyl3A (5AE6 chain B). The structure of PcBxl3 with xylose bound in the catalytic center was also determined. The data collection statistics and structural refinement statistics are summarized in Table 1.

PcBxl3 consists of three domains: (β/α)₈ domain, (α/β)₆-sandwich domain, and fibronectin III (FnIII)-like domain. The overall structure and order of these domains (illustrated in Fig. 2) are the same as those of TrXyl3A. The FnIII-like domain is connected to the active domain by a linker, as in other GH3 enzymes. There is a single molecule of PcBxl3 in the asymmetric unit, whereas there is a protein dimer in the case of TrXyl3A. N-Acetylgalcosamine moieties were found at Asn17, Asn40, Asn74, Asn279, Asn286, Asn398, Asn432, and Asn437 of free PcBxl3.

Focusing on the N-terminal region, an extra loop containing the α-helix is found near the active center of TrXyl3A, as shown in Figure 3A, which is consistent with the results of the
secondary structure prediction. In contrast, both enzymes were found to have the C-terminal at the same location even though the secondary structure comparison predicted that \textit{TrXyl3A} would have a longer C-terminal region.

**Comparison of the active center**

The catalytic residues of GH3 enzymes are glutamic acid and aspartate (17–19). Superposition of our enzymes on \textit{\beta}-D-exohydrolase from \textit{Hordeum vulgare} (\textit{HvExoI}, 1IEX) suggests that \textit{PcBxl3} has Glu470 as a possible acid/base amino acid and Asp267 as a catalytic nucleophile, while the corresponding residues in \textit{TrXyl3A} are Glu492 and Asp291. The conformation of Glu470 (\textit{PcBxl3}) is the same in the native and liganded structures of \textit{PcBxl3}, but Glu492 (\textit{TrXyl3A}) has different conformations in native and liganded \textit{TrXyl3A}.

As shown in Figure 3B, both Bxs have a pocket at subsite -1. The binding mode of a xylose residue at subsite -1 is important for substrate recognition and to maintain the substrate in a suitable conformation during the catalytic reaction. There are three important interactions, as illustrated in Figure 3, C and D: the interactions with the C2, C3, and C4 hydroxyl groups (O2, O3, and O4, respectively), the interaction with C5, and the interactions with the \alpha- and \beta-faces of the xylose ring, which are named according to the usual rules (20), as shown Figure 3E. The hydroxyl groups interact with hydrophilic amino acids. For instance, Asp267 in \textit{PcBxl3} is located at a distance of 2.7 Å from O2 of the xylose moiety bound at subsite \textbf{-1}, while Arg166 and Asp291 in \textit{TrXyl3A} are located at distances of 2.7 Å and 2.8 Å, respectively. In addition, Arg143 and His184 are located at distances of 3.0 and 2.4 Å from O3 of the xylose residue, respectively. Furthermore, the amino acids near the C4 hydroxyl group (O4) of the xylose residue bound at

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**Table 1**

Data collection and refinement summary

| Data                          | \textit{PcBxl3} | \textit{PcBxl3} bound with X | \textit{TrXyl3A} bound with thioxylobiose |
|------------------------------|-----------------|-------------------------------|-------------------------------------------|
| Wavelength                   | 1.000           | 1.000                         | 1.038                                     |
| Resolution range             | 44.44–2.54 (2.631–2.54) | 44.08–3.08 (3.19–3.08) | 28.89–2.1 (2.175–2.1)                     |
| Space group                  | P 21 21 21     | P 21 21 21                   | P 21 21 21                               |
| Unit cell (a/b/c)            | 79.667/91.292/107.082 | 79.367/91.071/106.012       | 100.244/202.443/82.441                   |
| Total reflections            | 52,707 (5163)   | 29,463 (2896)                 | 192,986 (19,158)                         |
| Unique reflections           | 26,401 (2587)   | 14,750 (1449)                 | 98,434 (9738)                            |
| Multiplicity                 | 2.0 (2.0)       | 2.0 (2.0)                     | 2.0 (2.0)                                |
| Completeness (%)             | 99.94 (99.92)   | 99.91 (99.93)                 | 99.79 (99.85)                            |
| \textit{R}_{merge}           | 0.08141 (0.3395) | 0.09164 (0.3472)             | 0.04487 (0.1965)                        |
| Reflections used in refinement | 1262 (127)    | 720 (71)                      | 4910 (478)                               |
| \textit{R}_{work}            | 0.1835 (0.2554) | 0.2005 (0.2501)              | 0.1761 (0.2088)                         |
| \textit{R}_{free}            | 0.2369 (0.3252) | 0.2562 (0.3378)              | 0.2123 (0.2480)                         |
| Number of nonhydrogen atoms | 6140            | 6161                          | 13,404                                   |
| Macromolecules               | 5623            | 5609                          | 11,971                                   |
| Ligands                      | 118             | 339                           | 482                                      |
| Solvent                      | 399             | 213                           | 951                                      |
| RMS (bonds)                  | 0.004           | 0.003                         | 0.007                                    |
| RMS (angles)                 | 1.06            | 0.73                          | 1.15                                     |
| Ramachandran favored (%)     | 95.55           | 93.79                         | 96.65                                    |
| Ramachandran allowed (%)     | 3.51            | 5.4                           | 3.16                                     |
| Ramachandran outliers (%)    | 0.94            | 0.81                          | 0.2                                      |
| Rotamer outliers (%)         | 0.0             | 0.0                           | 0.0                                      |
| Average B-factor             | 34.54           | 42.34                         | 22.87                                    |
| Macromolecules               | 34.16           | 41.24                         | 22                                       |
| Ligands                      | 34.7            | 35.94                         | 26.23                                    |
| Solvent                      | 52.37           | 64.63                         | 38                                       |
| PDB code                     | 7VC6            | 7VC7                          | 5AE6                                     |

Statistics for the highest-resolution shell are shown in parentheses. This table was created using the Phenix program.
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Comparison of kinetic parameters

Kinetic analysis was conducted to examine the substrate specificities toward xylooligosaccharides. As expected from the structural differences between PcBxl3 and TrXyl3A, the kinetic parameters are significantly different (Table 2), i.e., the values of $k_{\text{cat}}$ toward $X_2$, $X_3$, $X_4$, and $X_5$ are in range from 1.66 to 2.05 s$^{-1}$ for PcBxl3, while those of TrXyl3A range from 0.34 to 0.82 s$^{-1}$, about a quarter of those of PcBxl3. The $k_{\text{cat}}$ of TrXyl3A is DP-dependent, increasing up to DP 4 and then decreasing at DP 5. In contrast, the $K_M$ values of PcBxl3 are higher than those of TrXyl3A. The measured $K_M$ values of PcBxl3 range from 0.74 to 1.21 mM, while those of TrXyl3A range from 0.024 to 0.09 mM, a tenth of those of PcBxl3. Thus, these two enzymes have very different catalytic characteristics.

The calculated $k_{\text{cat}}/K_M$ values, representing catalytic efficiency, indicate that TrXyl3A is two to ten times more efficient than PcBxl3, though direct comparison is difficult since the enzymes have different reaction characteristics. Thus, for further evaluation of their substrate specificity, the DP dependence of $k_{\text{cat}}/K_M$ was investigated (Fig. 4). The DP of xyloooligosaccharides had little effect on the $k_{\text{cat}}/K_M$ value of PcBxl3 compared with that of TrXyl3A. The $k_{\text{cat}}/K_M$ of TrXyl3A, in contrast, increased with increasing DP of the substrate. Longer xyloooligosaccharides than $X_2$ were better substrates for TrXyl3A and $X_3$ was the best substrate. Thus, the kinetics parameters suggest that PcBxl3 degrades different xyloooligosaccharides equally well, whereas TrXyl3A preferentially degrades longer oligosaccharides.

Subsite affinities

The subsite affinities (23) of both Bxl3 were calculated from the kinetic parameters. In the case of exo-type enzymes such as

Figure 3. The structure of the active center. A, the surface of PcBxl3 and TrXyl3A. PcBxl3 and TrXyl3A are colored sky blue and salmon, respectively. Xylose and thioxylobiose are represented as green (carbon), red (oxygen), and yellow (sulfur) sticks. The red circle shows the extra loop of TrXyl3A. B, the hole-shaped active center. Representative small compounds are the same as in A. The surfaces of PcBxl3 and TrXyl3A are shown in sky blue and salmon, respectively. The red region is the extra loop of TrXyl3A. C, the hydrophilic residues within 3.0 Å from substrates and hydrophobic residues around substrates are shown as sticks. Oxygen, nitrogen, and sulfur are colored red, blue, and yellow, respectively. Carbon is colored green. D, the ligand–protein diagrams were created using LigPlot+ (63). E, the definition of α- and β-faces of the xylose ring is based on the proposed rule (20).
Bxl, the ratio of \( \frac{(k_{\text{cat}}/K_M)_{n+1}}{(k_{\text{cat}}/K_M)_n} \) reflects the binding affinity at subsite \( n \). Based on the maximum value of \( k_{\text{cat}}/K_M \) of PcBxl3 and TrXyl3A was assumed to be 2.05 and 0.82 s\(^{-1}\), respectively. The results are shown in Figure 5. We attempted to calculate the affinities of subsites \(-1\) and \(+1\) according to the methods of Hiromi and coworkers (24, 25), but without success. To obtain the subsite affinity at subsite \(-1\), it is necessary to assume that there are two binding modes, productive and nonproductive (25). However, this would not be the case for PcBxl3. On the assumption of two binding modes, the subsite affinity at subsite \(-1\) can be derived from \( k_{\text{cat}} \) and the affinities of subsites \(+2\) to \(+4\) (25). However, this does not work in the present case, because there are very weak affinities at subsite +3 and +4 in PcBxl3. Therefore, since our focus here is on the relationship between DP and Bxl function for DP = 2 or more, we used the sum of the affinities of subsites \(-1\) and \(+1\) as a parameter for comparison. As expected from the results for other glycosidases, the active center (sum of subsites \(-1\) and \(+1\)) has a higher affinity than the sum of the affinities of other subsites. TrXyl3A shows higher affinity at the active center than PcBxl3, and the difference is about 1 kcal/mol, which is consistent with the structural findings.

Interestingly, subsite +2 of TrXyl3A has a binding energy of 1 kcal/mol, which is typical for subsites of other GH (6, 25–29), whereas the binding energy at this subsite of PcBxl3 was very low, and subsite +3 and +4 also showed little affinity. These results indicate that PcBxl3 has at most two substrate-binding subsites, i.e., subsite \(-1\) and subsite \(+1\), whereas TrXyl3A has three, i.e., subsites \(-1\), \(+1\), and \(+2\).

**Docking simulation**

Next, the affinities of xylose and \( X_2 \) (PcBxl3 and TrXyl3A), and \( X_3 \) (TrXyl3A) were estimated by docking simulation; the results are summarized in Table 3 and visualized in Figure 6. While some simulations showed different modes of binding, most of the highest scores were recorded for the same binding mode as that of the xylose residue in the crystal structure of PcBxl3 and as that of thioxylobiose in the crystal structure of TrXyl3A, supporting the reliability of the simulation results. In the case of PcBxl3, \( X_2 \) showed the highest score, \(-6.3\) kcal/mol. In the case of TrXyl3A, \( X_2 \) and \( X_3 \) showed the highest score, \(-7.9\) and \(-8.9\) kcal/mol, respectively. The difference between the

**Figure 4. Relationships between substrate specificity and substrate DP value.** The ratio of the value of \( (k_{\text{cat}}/K_M)_n \) to that of \( (k_{\text{cat}}/K_M)_2 \) was calculated (see Table 1). Blue bars, PcBxl3; red bars, TrXyl3A.

### Table 2

| Parameters | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( K_M \) (mM) | \( k_{\text{cat}}/K_M \) (mM\(^{-1}\)s\(^{-1}\)) | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( K_M \) (μM) | \( k_{\text{cat}}/K_M \) (mM\(^{-1}\)s\(^{-1}\)) |
|------------|-----------------|--------------|------------------|-----------------|--------------|------------------|
| \( X_2 \)  | 1.81 ± 0.05     | 0.90 ± 0.06  | 2.0 ± 0.2        | 0.34 ± 0.01     | 90 ± 10      | 3.7 ± 0.6        |
| \( X_3 \)  | 1.80 ± 0.06     | 1.14 ± 0.08  | 1.6 ± 0.2        | 0.39 ± 0.01     | 24 ± 5       | 16 ± 4           |
| \( X_4 \)  | 2.05 ± 0.05     | 0.74 ± 0.04  | 2.8 ± 0.2        | 0.82 ± 0.03     | 70 ± 10      | 12 ± 2           |
| \( X_5 \)  | 1.66 ± 0.15     | 1.21 ± 0.25  | 1.4 ± 0.3        | 0.56 ± 0.01     | 66 ± 3       | 8.5 ± 0.5        |

The final concentrations of PcBxl3 and TrXyl3A were 30.2 nM and 26.6 nM, respectively. The reaction solution contained 50 mM sodium acetate buffer (pH 5.0). The reaction was performed at 30 °C for 30 min and stopped by heating the mixture at 95 °C for 5 min. The amount of released xylose was determined using HPLC with a Corona CAD detector.
scores of the X₂ and X₃ binding modes is -1.0 kcal/mol, which is consistent with the subsite affinity in Figure 5, while the difference between the scores of the Xyl and X₂ binding modes is -1.7 kcal/mol.

Comparing the scores of the xylose and X₂ binding modes of Pcbxl3 and TRXyl3A, the score of Pcbxl3 is about a half of that of TRXyl3A. As shown in Figure 6B, only O₃ of the xylose residue at subsite +1 can have a hydrophilic interaction with Tyr407 (Pcbxl3) and Tyr429 (TRXyl3A). Further, as shown in Figure 6C, less than four atoms of the xylose residue at subsite +1 interact with Pcbxl3, whereas six atoms of xylose with the residue at subsite +1 interact with TRXyl3A. Thus, the difference between the scores is thought to be mainly due to the difference in number of hydrophobic interactions at subsite +1.

Focusing on TRXyl3A docked with X₃ in Figure 6B, Leu17 is located at a distance of 3.0 Å from C5 of the xylose moiety at subsite +2. Additionally, two water molecules on the surface of the extra loop are placed near the xylose moiety, at a distance of 2.9 Å. They may have a role in binding the substrate, because the results of docking simulation without water gave a significantly lower score (data not shown). These results support that the idea TRXyl3A contains subsite +2 and suggest that the extra loop is important for binding longer oligosaccharides.

**Discussion**

**Comparison of GH3 enzymes**

The substrate specificity of GH3 enzymes is mainly determined by subsite -1, because exo-type enzymes must recognize and bind the residue at the nonreducing end throughout the reaction. Thus, subsite -1 of enzymes that have similar substrate specificities generally consists of the same or similar amino acid residues. Figure 7 shows the structure of the active center of four GH3 enzymes: Pcbxl3 (7VC7), TRXyl3A (5AE6),

![Figure 5. Summary of subsite affinities. Subsite affinities were calculated from kcat/Km in Table 1 by Hiromi’s method (23-25). Blue bars, Pcbxl3; red bars, TRXyl3A.](image)

**Table 3**

Scores from docking simulation

| Pcbxl3 | Affinity (kcal/mol) | TRXyl3A | Affinity (kcal/mol) |
|--------|---------------------|---------|---------------------|
|        | Xylose              | X₂      | Xylose              | X₂      | X₃      |
| Binding mode |                    |         | Binding mode |                    |         |         |
| 1       | -5.8                | -6.3    | 1                  | -6.2    | -7.9    | -8.9    |
| 2       | -5.6                | -6.3    | 2                  | -6.1    | -7.5    | -8.6    |
| 3       | -5.3                | -6.1    | 3                  | -5.4    | -7.3    | -8.2    |
| 4       | -4.9                | -5.9    | 4                  | -5.2    | -7.1    | -8.1    |
| 5       | -4.6                | -5.7    | 5                  | -5.1    | -6.9    | -7.9    |
| 6       | -4.1                | -5.1    | 6                  | -5.1    | -6.9    | -7.8    |
| 7       | -4.0                | -5.1    | 7                  | -4.9    | -6.8    | -7.0    |
| 8       | -3.9                | -5.0    | 8                  | -4.9    | -6.7    | -7.0    |
**Figure 6. Docking simulation.** The model structures of X₂ and X₃ were created using the SWEET2 server (66, 67). Docking simulation was done by Autodock Vina (64). Substrates are represented as green (carbon) and red (oxygen) sticks. A, the surface and sticks of PcBxl3 and TrXyl3A are shown in sky blue and salmon, respectively. The surface of the extra loop of TrXyl3A is red. The sticks and the extra loop are shown in red. B, hydrophilic residues near the substrate (within 3.0 Å) and hydrophobic residues around the substrates are shown as sticks. The colors are the same as in Figure 3B. Water molecules are shown as red asterisks. C, ligand–protein diagrams were created using LigPlot+ (63).

**TrCel3A (4I8D) (22) and HvExoI (1IEX) (17).** Most of the hydrophilic amino acids interacting with O₂, O₃, and O₄ are common among them. Whether they bind xylose or glucose, the modes of binding are conserved in the GH3 enzymes. A key difference between xylose and glucose is that xylose lacks C₆. As shown in Figure 3, Bxl has a tryptophan residue near C₅. Since Bgl do not have this tryptophan residue, there is sufficient space for C₆. Thus, the tryptophan residue appears to have a role not only in the hydrophobic interaction with C₅, but also in substrate selectivity. Furthermore, the environment at the α- and β-faces is different between Bxs and Bgls. As shown in Figure 3, both Bxs have a tyrosine residue on the side of the α-face and disulfide and methionine on the other side. Thus, there is a hydrophobicity gradient. As shown in Figure 8, TrCel3A has Ser384 on the side of α-face and Try237 and methionine on the other side, while HvExoI has Trp158 on the side of the α-face and two methionines on the side of the β-face. Bgls also interact with the sugar ring hydrophobically, but only methionine on the β-face is common, and there does not seem to be the same hydrophobicity gradient. These differences provide insight into the binding mode of xylose at subsite -1 of Bxs. Since xylose is more symmetric than glucose, differentiation of the two sides of the ring is more difficult. However, the β-face is more hydrophobic than the α-face because of the difference of axial and equatorial hydrogen at C₅. To recognize this difference, there is a gradient of hydrophobicity on both sides in GH3 Bxs. Based on these considerations, the three tyrosine residues in subsite -1 of TrXyl3A seem to have different roles. Tyr257 is conserved in GH3 Bgls and Bxs. In TrCel3A, the equivalent tyrosine is thought to have a hydrophilic interaction with O₂. As shown in Figure 6, Tyr257 can be located sufficiently near to O₂ for hydrogen bonding. Thus, this tyrosine may bind O₂. Tyr429 of TrXyl3A is thought to interact not only with the α-face of the xylose ring as shown in Figure 7, but also with O₃ of the xylose residue at subsite +1, depending on the orientation of the
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xylose residue, as shown in Figure 6. Since Tyr152 of TrXyl3A is located at a position corresponding to Phe129 of PcBxl3 (Fig. 3), this tyrosine contributes to the hydrophobic environment.

In contrast to subsite −1 in Bxls, the structures of subsite +1 in PcBxl3 and TrXyl3A are significantly different. In the crystal structure of PcBxl3, subsite +1 was not clearly identified, but that of TrXyl3A was clearly visualized in the crystal structure. The isoprimeverose (α-D-xylopyranose-(1→6)-D-glucopyranose derived from xyloglucan)-producing enzyme belonging to GH3 from Aspergillus oryzae has a shallow pocket adjacent to subsite −1, broadening it to subsite −1’ (30). The pockets of Lin1860 from Listeria innocua and BT_3567 protein from Bacteroides thetaiotaomicron, which degrade β-1,2-glucooligosaccharides, are also shallow (31, 32). These subsite structures are consistent with a wider range of substrate structures. In the case of Bgls degrading cellooligosaccharides, the glucose moiety at subsite +1 is sandwiched between two tryptophan residues; this is possible because the substrates have a straight chain without substitution. GH3 Bgls have a pocket large enough to bind disaccharides, while GH3 Bxls have a smaller pocket, and TrXyl3A has the extra loop that contributes to binding longer, substituted xylooligosaccharides.

Furthermore, it was suggested that TrXyl3A contains subsite +2 because the calculated affinity, +1 kcal/mol, is consistent with that of extra subsites in other enzymes (6, 25–29). The shape of subsites is also important. For example, in KmBgl3, the subsite specificity is restricted by the PA14 loop (7). In contrast, the extra loop of TrXyl3A is open. The space around O2 at subsite +1 of TrXyl3A can accept substitutions such as GlcA and arabinose. However, O3 is more restricted, and it was reported that TrXyl3A cannot degrade XA3XX (14), though an acetyl group at O3 is small enough to permit binding (21). Thus, wood-decaying fungi and soft-plant-decaying fungi have specific Bxls to optimize their xylan-degradation systems.

Different roles of tyrosine residues around the active center

Based on the above results and discussion, the three tyrosine residues in subsite −1 of TrXyl3A have different roles. Tyr257 is conserved in GH3 Bgls and Bxls. In TrCel3A, the corresponding tyrosine is thought to have a hydrophilic interaction with O2. As shown in Figure 6, TrCel3A is thought to interact not only with the α-face of the xylose ring as shown in Figure 7, but also with O3 of the xylose residue at subsite +1, depending on the orientation of the xylose residue (Fig. 6). Since Phe129 of PcBxl3...
(Fig. 3) is located at a position corresponding to that of Tyr152 of TrXyl3A, this tyrosine contributes to the hydrophobic environment.

**Relationship between kinetics and structure**

Subsite theory can account for the differences in the kinetic parameters of the enzymes studied here. Kinetic analysis revealed that the \( k_{cat} \) values of PcBxl3 are generally higher than those of TrXyl3A. This is probably because TrXyl3A has more subsites, which would increase the ratio of nonproductive binding. In contrast, the \( K_M \) values of TrXyl3A are one-tenth of those of PcBxl3, which translates into higher substrate affinity in general.

In summary, PcBxl3 degrades xylooligosaccharides with various DPs and prefers X2, based on the crystal structure. In contrast, TrXyl3A degrades longer xylooligosaccharides with specific substitutions (3, 14, 21).

**Xylan degradation strategies**

Xylanases produce various substituted xylooligosaccharides (3). Differences in fungal species, the characteristics of their enzymes, and the target xylan structures need to be considered to understand the overall xylan degradation system. Figure 8 shows the phylogenetic tree of 31 fungi based on the study by Floudas et al. (33), combined with information on fungal ecology and the numbers of GH10 and GH11 enzymes (33), as well as the presence/absence of GH3 Bxs and the presence/absence of the extra loop. Not all basidiomycetes have GH3 Bxs and among those that do, the enzyme seems to lack the extra loop. There appears to be no major difference between white-rot fungi (WR) and brown-rot fungi (BR). In contrast, Bxs from the ascomycetes T. reesei and Aspergillus niger (34) have the extra loop region.

Focusing on geological age, it seems likely that fungi and molds acquired GH3 Bxs and GH11 xylanases in the Carboniferous period. These Bxs may have lacked the loop region, such as \( T. \) reesei and Aspergillus niger (34) have the extra loop region.
systems in response the subsequent evolution of their target plants.

White-rot fungi have GH10 xylanases and favor hardwood substrates. Since the substitution pattern of glucuronic acid is distributed (4) and approximately one in two xylose resides is acetylated (36), acetylglucuronoxylan is resistant to degradation by xylanases (37), and acetyl xylan esterases play an important role (38). If GH10 xylanases and acetyl xylan esterases attack xylan, the final products would be xylose, X2 and 23-α-L-arabinofuranosyl-xylooligosaccharides. Brown-rot fungi also have GH10 xylanases, but favor softwood substrates, which include arabinoglucuronoxylan. The structure of arabinoglucuronoxylan is simpler than that of acetyl glucuronoxylan in that glucuronic acid substitution mostly occurs once every six xylose residues, and arabinose is located two residues away from a GlcA-substituted residue (39). Thus, GH10 digestion affords xylobiose and xylooligosaccharides substituted with GlcA or Ara at the nonreducing end (39). Bxl in this environment is mainly required to degrade these relatively small oligosaccharides after debranching. In contrast to wood-decaying fungi, soft-rot fungi and molds mainly use GH11 and GH30 xylanases.

In this paper, two types of fungal Bxls were characterized and compared with each other. Kinetic analysis taking account of the subsite structure and substrate DP could account for the different substrate specificities of PcBxl3 and TrXyl3A. Our results highlight the different fungal tactics employed to degrade xylan: wood-decaying basidiomycetes use Bxls, such as PcBxl3, that act efficiently on xylan structures from woody plants, whereas molds use Bxls that efficiently degrade xylan from grass. These findings provide new insight into our understanding into the fungal efficient xylan degradtion system.

Experimental procedures

Sequence analysis

Amino acid sequence of TrXyl3A was obtained from GenBank (CA932481.1, http://www.ncbi.nlm.nih.gov) (40). Amino acid sequence of PcBxl3 was obtained from Joint Genome Institute (JGI) MycoCosm (Protein ID: 2919526) (41). The Blastp server (https://blast.ncbi.nlm.nih.gov/Blast.cgi? PAGE=Proteins) was used for calculation of identity. The amino acid sequences were aligned using Clustal W (42). Secondary structures were predicted by ESPript using the alignment and the native form of TrXyl3A (43).

Protein preparations

P. chrysosporium strain K-3 was grown on Kremer and Wood medium (44) containing 2% cellulose (CF11; Whatman, Kent, UK) as the sole carbon source. Total RNA was extracted from approximately 100 mg of frozen mycelial powder using Isogen (Nippon Gene), and mRNA was purified from 1 μg of the total RNA using Oligotex-dT30 Super (Takara Bio), both according to the manufacturers’ instructions. Synthesis of first-strand cDNA from the mRNA was performed using a GenExRacer kit with SuperScript III reverse transcriptase (Invitrogen). The oligonucleotide primers, Pcxyn3_07481_3RACE (5’-GGTCGTCAGCCCATTTAAGCTAG-3’) and Pcxyn3_9257_5RACE (5’-GCGGGAACGAACTAGCA-GAGCTG-3’) for amplification of a cDNA fragment encoding PcBxl3, were designed based on the genomic sequences of P. chrysosporium available at Genome JGI. PCR was performed using KOD-Plus (TOYOBO). The cloned fragment was amplified with the gene-specific primers, Pcxyn3_9257_Meta (5’-ATGGTCGCCAGCCATTTAAGCTAG-3’) and Pcxyn3_9257_Meta (5’-GCTGCTATGTA-CACTCCGTGTTTGA-3’), and ligated into pPICZα A plasmid vector (Invitrogen) using EcoRI and NotI restriction recognition sites. After transformation of Pichia pastoris strain KM71H by electroporation, transformants were selected with Zeocin according to previous research (45-47).

pPICZα A plasmid vector encoding Trxyl3A between Xhol and NotI restriction recognition sites was optimized for P. pastoris and synthesized by Genscript. Subsequent procedures were as described above.

The recombinant proteins were produced using a 5 L jar fermenter (TSC-M5L; Takasugi Seisakusho) with methanol feed according to previous reports (45-47). The concentration of crude protein was quantified using Bradford Protein Assay (Bio-Rad) (48) using bovine serum albumin as standard. The yields of PbBxl3 and TrXyl3A were about 450 mg/L and 2500 mg/L, respectively. After 120 h, the medium was collected by centrifugation (30 min at 5000g), subjected to 100 kDa ultrafiltration, and concentrated using a 5 kDa filter (Merck Millipore).

The resulting solutions were purified by two-step column chromatography. For the characterization of PbBxl3 and TrXyl3A, Phenyl Toyopearl 650M (c.v. = 20.5 ml, Tosoh) was used for the first chromatography. After equilibration with 50 mM sodium acetate buffer (pH 5.0) containing 1 M ammonium sulfate (Wako), the enzymes were eluted with 50 mM sodium acetate buffer (pH 5.0). TSkel DEAE-5PW (c.v. = 3.3 ml, Tosoh) was used for further purification. After equilibration with 50 mM Tris-HCl buffer (pH 8.0), the enzymes were eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 1 M NaCl (Wako). The concentration of the purified proteins was quantified by NanoDrop2000 (Thermo Fisher Scientific).

For crystallization of PbBxl3, deglycosylation was conducted by endoglycosidase H from Streptomyces pilatus before the DEAE column chromatography. The pET 28 vector with the gene encoding endoglycosidase H was a generous gift from
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Professor Satoshi Kaneko. Transformation of Escherichia coli BL21(DE3) (New England Biolabs) was conducted according to the manufacturer’s instructions. After cultivation in LB medium, protein expression was induced with 100 μM IPTG at 37 °C. The medium containing cells was centrifuged at 10,000g for 10 min, and cell lysis was performed using BugBuster (Merck) according to the manufacturer’s instructions. The recombinant endoglycosidase H was purified using HisTrap HP column (GE). After equilibration with 20 mM Tris-HCl buffer (pH 7.0) containing 500 mM imidazole (Wako), the enzyme was eluted with 20 mM Tris-HCl buffer (pH 7.0). The protein solution of PcBx13, without denaturing, was mixed with 0.2 mg purified endoglycosidase H per 1 mg of PcBx13 and sodium acetate buffer (pH 6.0). The mixed solution was incubated at 37 °C for 24 h.

The protein used for crystallization of TrXyl3A was a kind gift from DuPont Industrial Bioscience. The protein was stored prior to crystallization experiments at 4 °C in a stock solution containing 149 mg/ml protein, 13% sorbitol, and 0.125% sodium benzoate in 0.1 M Sodium acetate (pH 5.0).

**Crystallization of Bxl3**

The crystallization of PcBx13 was conducted by sitting-drop vapor diffusion method using a 96-well sitting drop plate (Greiner). For crystallization, 0.5 μl of 10 mg/ml PcBx13 was mixed with 0.5 μl of the reservoir solution containing 0.2 M lithium sulfate, 0.1 M Tris (pH 8.5) and 40 v/v % PEG 400 (No.43 of JCSG + Suite, Qiagen). The plate was incubated at 293 K for 3 weeks. The reservoir solution for soaking contained 525 mM malic acid (pH 7.0, QIAGEN) and 20 v/v % PEG 3350 (Sigma-Aldrich). The mixture was incubated at 293 K for 1 week. The crystal was soaked in 40 v/v % PEG 400 (Sigma-Aldrich) and 100 mM X2 (Megazyme) for a short time.

The TrXyl3A protein stock solution was diluted to 10 mg/ml by adding 0.1 M sodium acetate buffer (pH 4.5) just prior to crystallization. Optimized TrXyl3A crystals for data collection were obtained by the hanging drop vapor diffusion method. Crystals for ligand-bound TrXyl3A data collection were obtained using the PACT screen (Qiagen) condition C4 (0.1 M PCB (pH 7.0) and 25% PEG 1500). 4-thioxyloboise was chemically synthesized according to the previous research (49). Soaking of 4-thioxylobose to the crystals was done by 1 h incubation of TrXyl3A crystals in 0.095 M PCB (pH 7.0) and 33% PEG 1500, with 14 mM 4-thioxylobose. Prior to data collection, the TrXyl3A crystals were briefly incubated in a cryoprotectant solution containing 30% PEG 3350 and 10% glycerol and then flash-frozen in liquid nitrogen.

**Data collection and structure determination**

The dataset of a TrXyl3A crystal soaked with 4-thioxylobose (to 2.1 Å resolution) was collected at beamline I911-5, at MAXII-lab, Sweden. All TrXyl3A diffraction data were processed using the data integration program Mosflm (50) and scaled using Scala in the CCP4i Software suite (51). The TrXyl3A 4-thioxylobose ligand structure was solved by molecular replacement (MR) using the program Phaser (52, 53) using the nonligated TrXyl3A structure (PDB ID: 5A7M, chain B) as search model.

Structure refinement of the TrXyl3A ligand-bound structure was performed using the program REFMAC5 (54) and 5% of the data was excluded from the refinement for cross-validation and R_free calculations (55). Throughout the refinement, 2mFo-DFc and mFo-DFc sigma A weighted maps (56) were generated and inspected so that the model could be manually built and adjusted in Coot (57). The statistics of structure refinement is shown in Table 1.

Diffraction experiments for PcBx13 crystals were conducted at the beamline of the Photon Factory (PF), High Energy Accelerator Research Organization. Diffraction data were collected on a Dectris PILATUS3 S 6M (Dectris). Crystals were cryocooled in a nitrogen gas stream to 95 K. The crystal data of free PcBx13 were integrated and scaled using XDS installed in PREMO. The data for the crystal soaked in X2 solution were integrated and scaled using XDS and Aimless in the CCP4i2 program suite. Structural determination of PcBx13 was conducted by molecular replacement with MOLREP in the CCP4i2 program suite. N-Acetyl-d-glucosamine, xylose, water molecules, and crystallization agents were modeled based on the electron density map and the coordination distances. The orientation of xylose molecule was determined by mimicking that of 5A7M at subsite –1. Refinement was conducted by Refmac5 in CCP4i2 (58) and Phenix.refine (59) in the Phenix program suite (version 1.18.2–3874, Lawrence Berkeley National Laboratory; 51).

The coordinates for the final TrXyl3A and PcBx13 structure models, and the structure-factor amplitudes, have been deposited at the Protein Data Bank (PDB) (60–62), with access codes 5AE6 (TrXyl3A), and 7VC6 and 7VC7 (PcBx13), respectively.

PyMOL (The PyMOL molecular Graphics System, version 2.2.3, Schrödinger, LLC) was used for structural drawings. Ligand-protein interaction was depicted using Ligplot+ (v.2.2) (63).

**Kinetics of Bxl3**

Xylobiose (X2), xylotriose (X3), xylotetraose (X4), and xylopentaose (X5) (Megazyme) were used as substrates. The reaction mixture consisted of enzyme, 5 mM sodium acetate buffer (pH 5.0), and various concentrations of xylooligosaccharides in the range from 50 μM to 5 mM. The concentrations of PcBx13 and TrXyl3A were 30.2 nM and 26.6 nM, respectively. The reaction was performed at 30 °C for 30 min and stopped by heating the mixture at 95 °C for 5 min to prevent the reaction from continuing during quantification. Measurement of the amount of released xylose was performed by high-performance liquid chromatography (LC-2000 series; Jasco) with tandem columns of SUGAR-KS802 and 801 (Showa Denko). The samples were manually injected. The column oven was set at 70 °C. A Corona CAD detector (ESA Biosciences) was used.

**Subsite theory**

Subsite theory is a simple theory describing the relationship between kinetics parameters and subsite affinities. The
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calculation for each subsite was conducted as described (23–25).

**Docking simulation**

Simulation was conducted using the Autodock Vina program (1.1.2) (64) in Chimera (version 1.14.0) (65). The model structures of X2 and X3 were built using the Sweet2 server (66, 67).

**Data availability**

The structures presented in this paper have all been deposited in the Protein Data Bank (PDB) with the following codes: 5AE6 (crystal structure of TrXyl3A bound with thioxylobose), 7VC6 (native form of PcBxl3), and 7VC7 (crystal structure of PcBxl3 bound with xylose).

**Supporting information**—This article contains supporting information (42).

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**Abbreviations**—The abbreviations used are: Bgl, β-glucosidase; Bxl, xylan 1,4-β-xylosidase; CAZy, Carbohydrate-Active enZymes; DP, degree of polymerization; FnIII, fibronectin III; GH, glycoside hydrolases; HvExol, β-D-endoxydrolase from Hordeum vulgare; JGI, Joint Genome Institute; KnBgl3, GH3 Bgl from Kluyveromyces marxianus; PcBxl3, GH3 Bxl from P. chrysosporium; PDB, Protein Data Bank; TrCel5A, GH3 Bgl from T. reesei; TrXyl3A, GH3 Bxl from T. reesei; UXX, 2,3-α-D-glucuronolylxylotriose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentaose; XA3, 1-α-L-arabinofuranosyl-xylose; XAC1, 1-α-L-arabinofuranosyl-β-D-xylosidase; XAXX, 2,3-α-D-arabinofuranosyl-xylotetraose; XA2XX, 2,3-α-D-arabinofuranosyl-xylotetraose; XX, 2,3-α-D-glucuronoxylotetraose.

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