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Key amino acid residues for the endo-processive activity of GH74 xyloglucanase

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ABSTRACT

Unlike endo-dissociative-xyloglucanases, *Paenibacillus* XEG74 is an endo-processive xyloglucanase that contains four unique tryptophan residues in the negative subsites (W61 and W64) and the positive subsites (W318 and W319), as indicated by three-dimensional homology modelling. Selective replacement of the positive subsite residues with alanine mutations reduced the degree of processive activity and resulted in the more endo-dissociative-activity. The results showed that W318 and W319, which are found in the positive subsites, are essential for processive degradation and are responsible for maintaining binding interactions with xyloglucan polysaccharide through a stacking effect.

1. Introduction

Xyloglucan is a hemicellulose polysaccharide found in plant cell walls [2] that has a β-1,4-linked glucose backbone with α-D-xylose attached to C6 of the glucose residues. In addition, some of the xylose residues are substituted to form oligomeric side-chains containing galactose, arabinose or fucose residues [27]. In plant cell walls, xyloglucan forms a cellulose–xyloglucan network via hydrogen bonds with cellulose microfibrils, and is thought to play a physiologically important role in cell definition, cell expansion, and regulation of plant growth and development [2].

Several enzymes are involved in xyloglucan degradation, including endo-β-1,4-glucanases ([8,11,13,15,19,28], reviewed in [10]). Some cellulases have been reported to hydrolyse not only cellulose but also xyloglucan as a substrate analogue [19]. However, many endo-β-1,4-glucanases have high glucanase activity for xyloglucan but not for cellulose or cellulose derivatives; these xyloglucan-specific β-1,4-glucanases have been designated specifically as xyloglucanases [7].

As reported previously, we have screened xyloglucan-degrading microorganisms from soil and identified several xyloglucan hydrolases [20,23–26]. For instance, the fungus *Geotrichum* sp. M128 produces two xyloglucan-specific glycoside hydrolases that belong to the glycoside hydrolase family 74 (GH74), oligoxyloglucan reducing-end-specific cellobiohydrolase (OXG-RCBH), and xyloglucan-specific endo-β-1,4-glucanase (XEG). OXG-RCBH has unique exo-type activity for xyloglucan and recognises the reducing end of xyloglucan [23]. By contrast, XEG cleaves the glycosidic bond of the unbranched glucose residues in the main chain of xyloglucan [24]. Structural analysis of XEG and OXG-RCBH revealed that the exo-activity of OXG-RCBH depends on a loop structure in the active site cleft, which is not present in XEG, and deletion of the loop structure converts the exo-activity of OXG-RCBH to endo-activity [21].

The Gram-positive bacterium *Paenibacillus* sp. strain KM21 also produces a xyloglucanase designated as XEG74 [26]. In the degradation of tamarind seed xyloglucan by endo-type xyloglucanase, XXXG, XLXG, XXLG and XLLG (G: unbranched D-Glc*p* residue, X: α-D-Xyl*p*-(1 → 6)-β-D-Glc*p*, L: β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)-β-D-Glc*p*) are the final products (Fig. S1 in the Supplemental Materials) [16,28]. Although both *Geotrichum* XEG and *Paenibacillus* XEG74 produce XXXG, XLXG, XXLG and XLLG as the final products of tamarind seed xyloglucan degradation, the modes of activity of *Geotrichum* XEG and *Paenibacillus* XEG74 are different. *Geotrichum* XEG acts as an endo-dissociative-type xyloglucanase, while *Paenibacillus* XEG74 has an endo-processive mode of action toward xyloglucan [26]. At the beginning of hydrolysis, both endo-dissociative- and endo-processive-type xyloglucanases act on the internal β-1,4-linked glucose backbone of xyloglucan.

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Subsequently, endo-dissociative-type enzymes repeat desorption from the polysaccharide and hydrolysis. Conversely, endo-processive-type enzymes hydrolyse progressively without desorption, and produce final products such as XXXG, XXLG, XLXG and XLLG. Therefore, the hydrolysis pattern of endo-processive-type enzymes resembles that of exo-type enzymes [11]. In contrast to endo-processive xyloglucanases, the processive activity of cellobiohydrolases (for example CBHI and CBHII of *Trichoderma reesei*) has been investigated extensively [5,6,17,18]. Both CBHI and CBHII have a tunnel-like active site for processive activity. However, Grishutin et al. discussed that endo-processive mode xyloglucanases are unlikely to have a tunnel-like active site structure capable of accommodating xyloglucan [11]. Therefore, it is unclear how endo-processive mode xyloglucanases gain processive activity.

In this study, we investigated the importance of tryptophan residues located in the active site cleft, as is characteristic of *Paenibacillus* XEG74, for endo-processive activity. We found that two tryptophan residues are vital for endo-processive-type xyloglucanase activity, and are responsible for the conversion of endo-processive-type *Paenibacillus* XEG74 activity into endo-dissociative-activity.

2. Materials and methods

2.1. Homology modelling of Paenibacillus XEG74

The structure of the catalytic domain of *Paenibacillus* XEG74 was theoretically determined by homology modelling using the SWISS-MODEL server (http://swissmodel.expasy.org) and the structure of *Clostridium thermocellum* Xgh74A (PDB code: 2CN2) [16] as a template. *Paenibacillus* XEG74 showed 59% identity with *Clostridium* Xgh74A.

2.2. Construction of Paenibacillus XEG74(CD) mutants

Xyloglucanase XEG74 from *Paenibacillus* sp. strain KM21 consists of three modules: a catalytic domain (CD; residues 1–736), an X2 module (residues 750–833), and a C-terminal CBM3 (carbohydrate-binding module 3; residues 850–932) that has the ability to bind cellulose [26]. The catalytic domain of XEG74 was expressed in *Escherichia coli* fused with a 6 × His-tag at its C-terminus (80 kDa) and will be referred to as XEG74(CD) henceforth. The DNA fragment encoding XEG74(CD) was amplified by PCR and cloned into a pET-28a (+) expression vector (Novagen, La Jolla, CA, USA). The primers used in this study are listed in the Supplemental Materials Table S1. The W61A, W64A, W318A, and W319A mutants were constructed using the QuikChange procedure (Stratagene, La Jolla, CA, USA) using the primers listed in Table S1 and the pET28a-XEG74(CD) clone as a template.

2.3. Expression and purification of Paenibacillus XEG74(CD) and Geotrichum XEG

XEG74(CD) expression vectors were transformed into *E. coli* BL21-CodonPlus (DE3) RP (Stratagene). To induce the expression of XEG74(CD), the transformants were cultured at 30 °C overnight in Overnight Express™ Instant LB medium (Novagen). The soluble intracellular protein was extracted using the BugBuster protein extraction reagent with Benzonase Nuclease HC (Novagen), and purified using a His SpinTrap (GE Healthcare, Buckinghamshire, UK) with elution buffer (20 mM sodium phosphate, 400 mM NaCl, 500 mM imidazole, pH 7.4). *Geotrichum* XEG was expressed in the *E. coli* BL21-CodonPlus (DE3) RP and purified as described previously [22].

2.4. Kinetic analysis of XEG74(CD) mutants

Kinetic parameters were determined using 2.5 μg enzyme/mL at xyloglucan concentrations ranging from 0.05 to 4 mg/mL in 20 mM sodium phosphate buffer (pH 6.0) at 45 °C for 15 min. The reducing sugar content was determined using a bicinchoninate assay [9] at 98 °C for 15 min. The Michaelis constants (K_m) and specific activities were calculated by non-linear regression of the Michaelis–Menten equation using GraphPad PRISM Version 5.0 (GraphPad Software, La Jolla, CA, USA).

2.5. Viscosimetric assay

Viscosimetric assays were performed as described previously [26]. Briefly, 100 μL diluted enzyme (80 ng/mL) were added to 1 mL 0.8% tamarind seed xyloglucan (Megazyme International Ireland Ltd., Bray, Ireland) in 20 mM sodium-phosphate buffer (pH 6.0) at 45 °C, and assayed at various time points (0.5, 2, 4, 8, and 24 h). The viscosity of reaction mixtures was determined using a Viscometer K-2283 200 (Kusano kagaku, Tokyo, Japan) at room temperature. The reducing sugar content was determined by bicinchoninate assay [9] at 98 °C for 15 min. The degree of hydrolysis at each time point was calculated by comparison with complete digestion of the substrate with excess enzyme and incubation time (designated as 100% hydrolysis).

2.6. Analysis of xyloglucan digestion products by gel-filtration chromatography

One hundred microliters of diluted enzyme (XEG74(CD) and its mutants: 4 μg/mL; XEG: 2 μg/mL) in 20 mM sodium phosphate buffer (pH 6.0) were added to 1 mL of 0.8% xyloglucan dissolved in ultra-pure water and incubated at 45 °C. After various incubation times (2, 3, 5, or 8 h), the reaction solution was applied to a Superdex Peptide 10/300 GL gel-filtration column (GE Healthcare, Buckinghamshire, UK) at 30 °C. Ultra-pure water was used as the column eluent with a flow rate of 1 mL/min. The system was driven with a pump (PC-2080, JASCO, Japan) and equipped with a refractive index detector (RI-2031, JASCO).

3. Results

3.1. Comparison of Paenibacillus XEG74 and Geotrichum XEG

For structural comparisons between endo-dissociative-type and endo-processive-type xyloglucanases, the structure of *Paenibacillus* XEG74(CD) was theoretically determined by homology modelling (Supplemental Data). The overall structure of *Paenibacillus* XEG 74(CD) was compared with that of *C. thermocellum* Xgh74A as complexes with substrate (XLLG and XXLG) [16] and *Geotrichum* sp. M128 XEG [22] (Fig. 1A). The active site cleft structure of *Paenibacillus* XEG74(CD) was also compared with that of *Geotrichum* XEG (Fig. 1B). Although these enzymes have similar overall structures, *Paenibacillus* XEG74(CD) has four characteristic tryptophan residues (W61, W64, W318, and W319) around the active site cleft. W61 and W64 are found in the "negative" subsites [4]. In contrast, W318 and W319 are found in the "positive" subsites. *Geotrichum* XEG lacks these tryptophan residues, suggesting that the difference between endo-dissociative- and endo-processive activities is derived from the presence of these tryptophan residues.

3.2. Point mutation analysis of Paenibacillus XEG74

To confirm the importance of tryptophan residues W61, W64, W318, and W319 for endo-processive-type xyloglucanase activity,

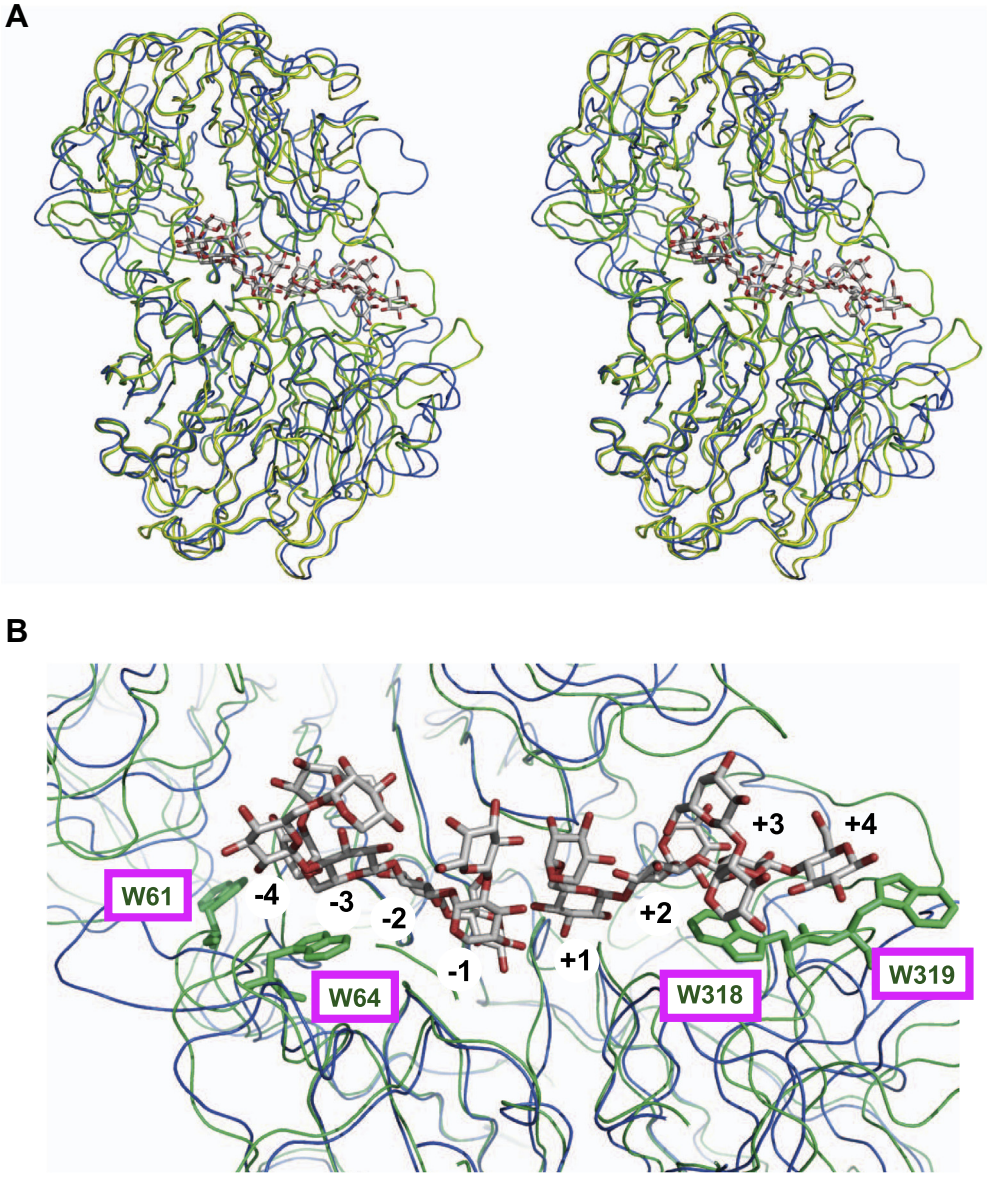

Fig. 1. Modelling of endo-dissociative- and endo-processive-type xyloglucanase structures. (A) Schematic drawing (divergent "wall-eyed" stereo) of structures of *Clostridium thermocellum* xyloglucanase Xgh74A (yellow), *Geotrichum* XEG (blue) and *Paenibacillus* XEG74(CD) (green). (B) Comparison between the active sites of *Geotrichum* XEG (blue) and *Paenibacillus* XEG74(CD) (green) is shown. Two substrate molecules, XLLG and XXLG, and four tryptophan residues of *Paenibacillus* XEG74(CD), W61, W64, W318 and W319, are shown as stick models.

we constructed four *Paenibacillus* XEG74(CD) variants, W61A, W64A, W318A, and W319A by site-directed mutagenesis. The *Paenibacillus* XEG74(CD) W-to-A substitution mutants were expressed in *E. coli*, purified, and their xyloglucan hydrolysis activities were analysed. All of these mutants showed xyloglucan hydrolysing activity, indicating that these tryptophan residues were not necessary for xyloglucan hydrolysis (Table 1). The K_m values for xyloglucan were increased in the XEG74(CD) W-to-A substitution mutants, especially in the W318A mutant, which exhibited a K_m value approximately twice that of the wild-type enzyme. Interestingly, however, XEG74(CD)-W318A also showed a high specific activity for xyloglucan.

We then analysed the effects of these mutations on the activity of *Paenibacillus* XEG74(CD) by studying the decrease in xyloglucan viscosity compared to the release of reducing sugars (Fig. 2). Endo-dissociative-type xyloglucanases reduce the viscosity of xyloglucan more rapidly than endo-processive-type xyloglucanases [26]. This drastic reduction in viscosity is caused by random depolymerisation of the xyloglucan backbone. Compared to the XEG74(CD) wild-type, mutations in W61 and W64 had no effect on the decrease in xyloglucan viscosity. However, in the case of the W318A and W319A mutants, the viscosity of xyloglucan was

Table 1
The kinetic parameters of the XEG74(CD) mutants.

	V_{max} (U/mg protein)	K_m (mg/ml)	k_{cat} (s^{-1})
XEG74(CD)	36.8	0.96	49.2
XEG74(CD)-W61A	40.7	1.30	54.3
XEG74(CD)-W64A	48.1	1.50	64.3
XEG74(CD)-W318A	54.0	2.18	72.0
XEG74(CD)-W319A	49.3	1.46	65.8

One unit was defined as the amount of enzyme that released 1 μmol of glucose equivalents as reducing sugars from xyloglucan per minute

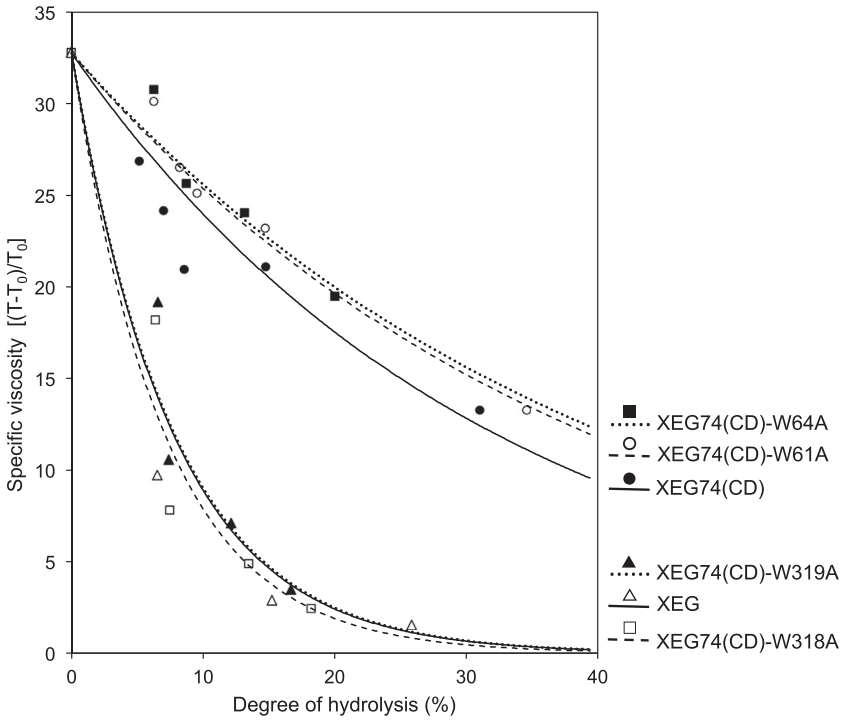

Fig. 2. Viscosimetric analysis of xyloglucan-hydrolysis products. Tamarind seed xyloglucan was digested with *Paenibacillus* XEG74(CD) (closed circles), XEG74(CD)-W61A (open circles), XEG74(CD)-W64A (closed squares), XEG74(CD)-W318A (open squares), XEG74(CD)-W319A (closed triangles) or *Geotrichum* XEG (open triangles) for 0.5–24 h. The specific viscosity and hydrolysis ratio of partial hydrolysis products were measured. T_0 is the flow time measured for the sodium-phosphate buffer, and T is the flow time of the reaction mixture incubated with xyloglucanases.

decreased quite rapidly (Fig. 2). This pattern is typical for endo-dissociative-type xyloglucanases, including *Geotrichum* XEG.

Next, xyloglucan was incubated with *Paenibacillus* XEG74(CD), W-to-A mutants, or *Geotrichum* XEG, and after various incubation times the degradation products were analysed by gel-filtration chromatography. In the case of *Paenibacillus* XEG74(CD) and its W61A or W64A mutant, the production of mid-range molecular weight compounds (retention time: about 9–12 min) was negligible. However, the final degradation products, such as XXXG, XLXG, XXLG, and XLLG, were detected as early as the initial stage of the reaction (Fig. 3B). Although wild-type *Paenibacillus* XEG74(CD) produced only final degradation products, the W61A and W64A mutants accumulated tetradeca-oligosaccharides, including XXXG and XXXG. In the case of the W318A and W319A mutants, the formation of mid-range molecular weight products was detected at similar levels to the endo-dissociative-type *Geotrichum* XEG. The accumulation of tetradeca-oligosaccharides was observed for the W318A mutant, although not for the W319A mutant.

From these results, we concluded that the activity of *Paenibacillus* XEG74(CD) was altered from an endo-processive-type to an endo-dissociative-type activity by mutation at W318 or W319, both of which are found in the positive subsites.

We also constructed double alanine substitution mutant enzymes, XEG74(CD)-W61A/W64A and XEG74(CD)-W318A/W319A. The hydrolysis pattern of the W318A/W319A double mutant closely resembled those of the W318A mutant and *Geotrichum* XEG (Figs. S2 and S3). In xyloglucan hydrolysis assays, the solution viscosity was reduced more rapidly using the W61A/W64A double mutant than was observed for the *Paenibacillus* XEG74(CD) wild-type, the W61A mutant, or the W64A mutant (Fig. S2, Fig. 2), and little formation of mid-range molecular weight products was detected (Fig. S3), indicating that mutations in W61 and W64 had synergistic effects on the mode of action of endo-processive-type xyloglucanase activity. These results demonstrated that although W61 or W64 is dispensable for endo-processive xyloglucanase activity, these residues contribute slightly to endo-processive activity and/or substrate recognition.

4. Discussion

In this study we performed homology modelling to determine a theoretical structure of the endo-processive-type xyloglucanase *Paenibacillus* XEG74(CD). Comparison of the active site structure with that of *Geotrichum* XEG allowed the identification of key amino acid residues involved in the distinction between endo-processive-type activity and endo-dissociative-type activity. *Paenibacillus* XEG74 has four tryptophan residues located in the active site cleft. It has been reported that some aromatic amino acid residues, and especially tryptophan residues, have the potential to participate in enzyme-substrate interactions by "stacking effects" [16]. We focused on these tryptophan residues that may be stacked with the glucose residue of the xyloglucan oligosaccharide. Mutation of the positive subsite residues W318 and/or W319 altered the activity of *Paenibacillus* XEG74(CD) from endo-processive to endo-dissociative-type. It is surprising that this alteration was due to a single amino acid substitution. This suggests that tryptophan residues in the positive subsites, and not the negative subsite residues W61 and W64, are responsible for maintaining the binding of polysaccharide in the active site after release of the xyloglucan-oligosaccharide product. This binding facilitates the motion of the enzyme along the polysaccharide chain to another catalytic event (Fig. 4). This may have implications on the directionality of processivity. Without W318 and/or W319, the enzyme leaves the substrate and is re-bound randomly along the backbone, resulting in endo-dissociative-type hydrolysis. By comparison to the W319A mutant, the W318A mutant caused a reduction in xyloglucan viscosity more rapidly and produced a large amount of mid-range

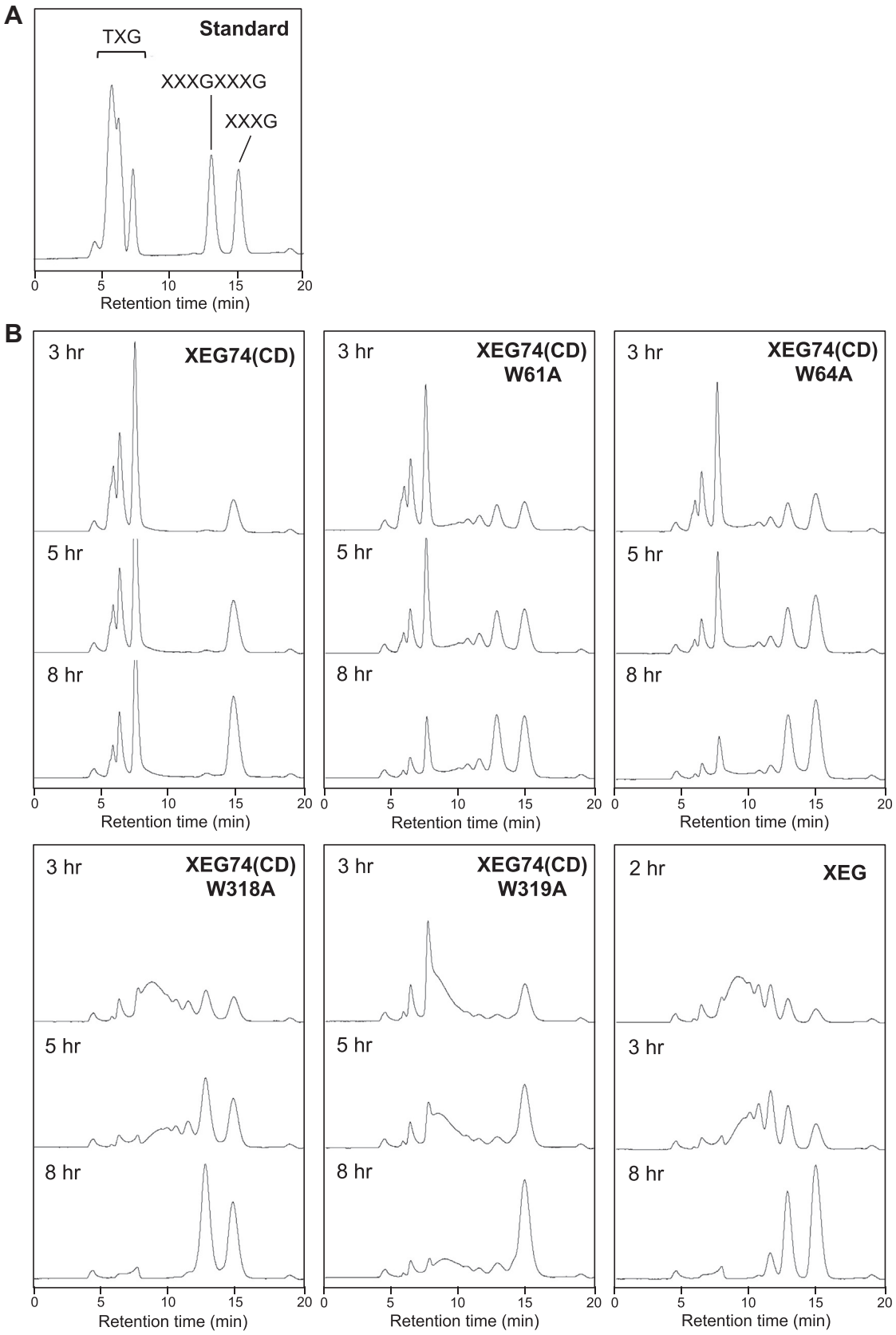

Fig. 3. Gel-filtration analysis of xyloglucan degradation products. (A) Tamarind seed xyloglucan (TXG) and xyloglucan-derived oligosaccharides including XXXG and XXXGXXXG were used as the standards. (B) Tamarind seed xyloglucan was incubated with *Paenibacillus* XEG74(CD), XEG74(CD) W-to-A substitution mutants, and *Geotrichum* XEG for various incubation periods. The partially hydrolysed xyloglucan products were applied to a gel-filtration column.

molecular weight products (Figs. 2 and 3). These results indicate that although both W318 and W319 are vital for endo-processive mode xyloglucanase activity, W318 is more important in processive-hydrolysis than W319.

We also attempted to convert endo-dissociative-type xyloglucanase *Geotrichum* XEG to endo-processive-type activity by introduction of active site cleft point mutation in *Geotrichum* XEG. Asparagine 326 of *Geotrichum* XEG (corresponding to W318 of *Paenibacillus* XEG74) was mutated to a tryptophan residue. However, this mutation did not affect the mode of activity (data not shown), suggesting that two or more aromatic amino acid residues are necessary to convert endo-dissociative-type enzymes into endo-processive-type enzymes.

A phylogenetic tree of GH74 xyloglucanases shows that they can be divided into three groups based on their mode of action: exo, endo-dissociative, and endo-processive (Fig. 5A). Endo-dissociative enzymes, such as *Geotrichium* XEG, and exo-type enzymes, such as *Geotrichum* OXG-RCBH [23] and *Aspergillus* OREX [1], lack tryptophan residues analogous to W61, W64, W318, and W319 of *Paenibacillus* XEG74 (Fig. 5B). *Streptmyces* GH74B [13], an endo-dissociative-type xyloglucanase, has three tryptophan residues analogous to W61, W64 and W318, but lacks a tryptophan residue corresponding to W319. The endo-processive-type enzymes *Phanerochaete* Xgh74B [15], *Streptmyces* GH74A [13], and *Thermobifida* Xeg74 [14, and our personal data (not shown)] have two tryptophan residues corresponding to W318 and W319, suggesting that both W318 and W319 are essential for the endo-processive activity of GH74 xyloglucanases. Although the modes of action of *Streptmyces* Sco6545 [8], *Aspergillus* EglC [12], *Trichoderma* Cel74A [3], and *Clostridium* Xgh74A [16] have not been

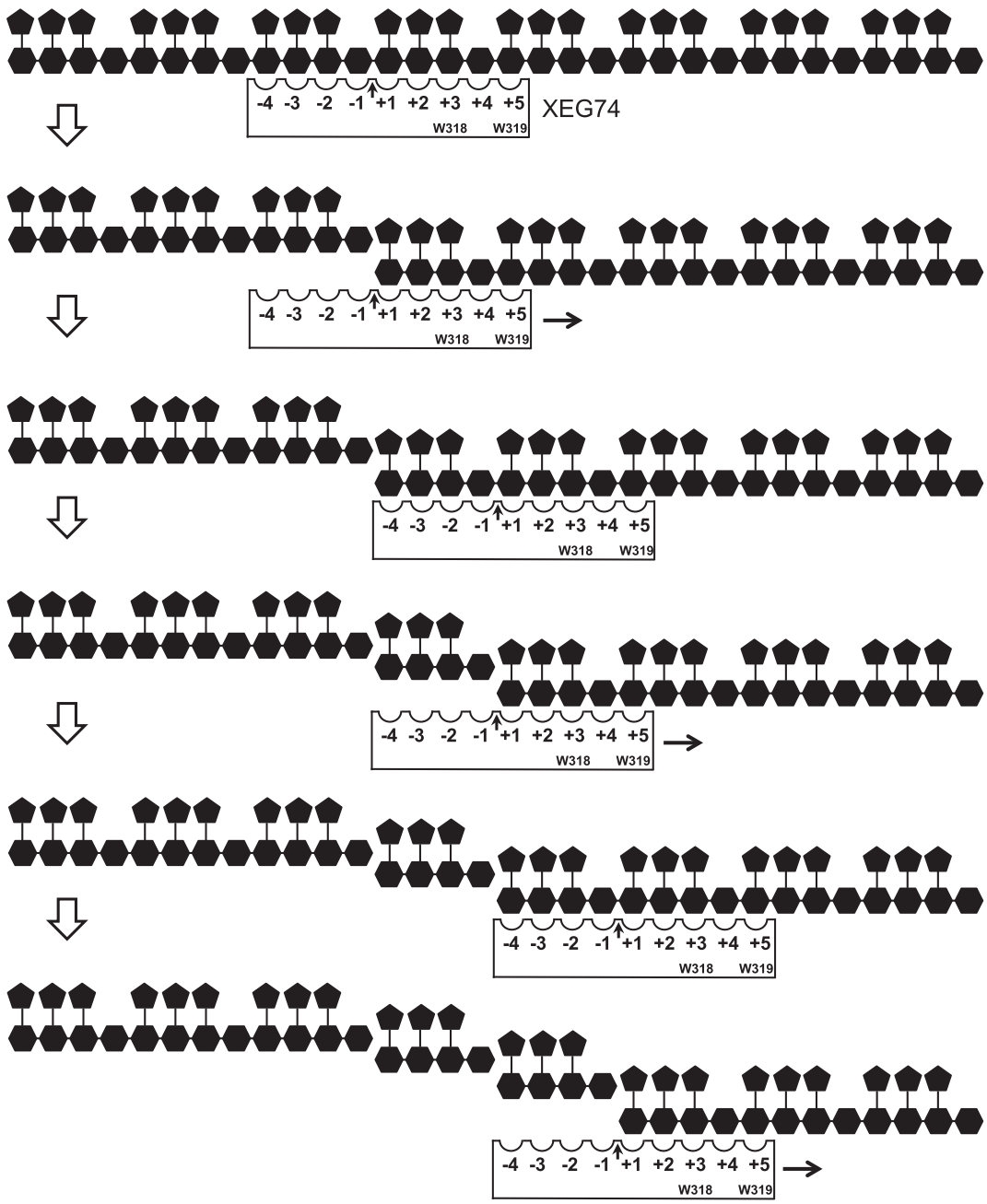

Fig. 4. The mode of activity of endo-processive-type xyloglucanase XEG74 is shown with tamarind seed xyloglucan as a substrate. Filled hexagons represent a glucose residue. Filled pentagons represent a xylose residue.

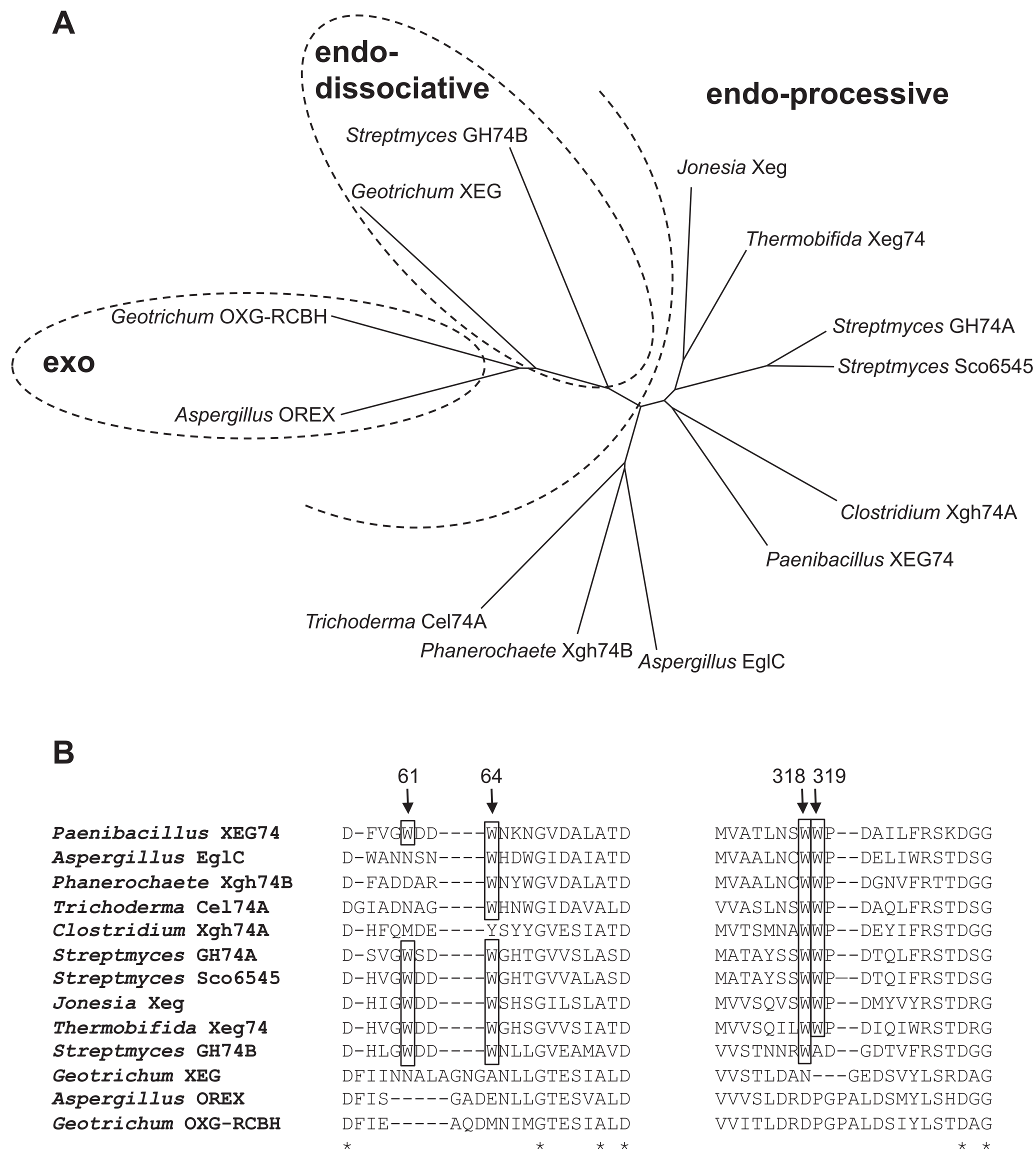

Fig. 5. Phylogenetic tree and sequence alignment of GH74 xyloglucan hydrolases (the amino acid sequence of catalytic domains). The sequence alignment was calculated using ClustalW (http://clustalw.ddbj.nig.ac.jp/), and the phylogenetic tree was constructed using FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). GenBank accession numbers are as follows: *Aspergillus niger* EglC, AAK77227.1; *Aspergillus nidulans* OREX, EAA64249.1; *Clostridium thermocellum* Xgh74A, CAE51306.1; *Geotrichum* sp. M128 OXG-RCBH, BAC22065.1; *Geotrichum* sp. M128 XEG, BAD11543.1; *Jonesia* sp. DSM 14140 Xeg, CAD58415.1; *Paenibacillus* sp. KM21 XEG74, BAE44527.1. *Phanerochaete chrysosporium* Xgh74B, BAF95189.1; *Streptmyces avermitilis* GH74A, BAC69567.1; *Streptmyces avermitilis* GH74B, BAC70285.1; *Streptmyces coelicolor* Sco6545, CAA20642.1; *Thermobifida fusca* Xeg74, AAZ55647.1; *Trichoderma reesei* Cel74A, AAP57752.1. The phylogenetic tree is shown in (A). The sequence alignments around W61 and W64 and around W318 and W319 are shown in (B). Conserved amino acids are indicated with asterisks. The positions of W61, W64, W318, and W319 of *Paenibacillus* XEG74 are indicated by arrows, and conserved tryptophan residues are indicated in boxes.

reported, tryptophan residues analogous to W318 and W319 of *Paenibacillus* XEG74 are conserved in these enzymes (Fig. 5B). It is therefore likely that these enzymes exhibit endo-processive activity.

Interestingly, several microorganisms contain both endo-dissociative- and endo-processive xyloglucanases [13,26]. Our future efforts will focus on how microorganisms use endo-dissociative- or endo-processive-type xyloglucanases during plant cell wall degradation. Because degradation of xyloglucan is an important issue in saccharification of lignocellulosic biomass [10], these studies will contribute to the knowledge of effective biomass-saccharification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.03.023.

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