

Both Substrate Hydrolysis and Secondary Substrate Binding Determine Xylanase Mobility as Assessed by FRAP

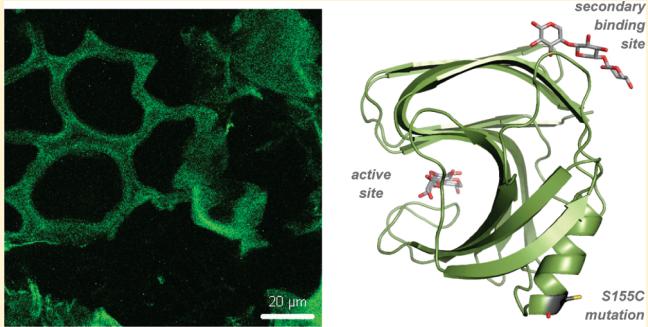
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Supporting Information

ABSTRACT: Xylanases (EC 3.2.1.8) are enzymes that can hydrolyze the xylan backbone internally. Therefore, they are important for biomass breakdown and they are also often added in various biotechnological applications. In this study, the relationship between their substrate binding affinity and hydrolysis, on the one hand, and their movement over natural substrates, on the other hand, was investigated. Fluorescence recovery after photobleaching (FRAP) experiments using different *Bacillus subtilis* xylanase A (XBS) mutants were conducted on water-unextractable wheat flour arabinoxylan (WU-AX) and insoluble oat spelt xylan (OSX). To assess the importance of substrate hydrolysis, FRAP of a catalytically inactive mutant was compared to that of the wild-type enzyme. For the wild-type enzyme, substrate binding and a complete recovery of fluorescence after photobleaching was observed on both substrates. For the inactive mutant, however, substrate binding but no fluorescence recovery was observed on WU-AX, while very slow recovery was observed on OSX. Furthermore, the importance of substrate binding to a secondary xylan binding site (SBS) for enzyme mobility was studied by testing two mutants with a modified SBS (NS4W-N141Q and G56A-T183A-W185A) that showed different behavior on the tested substrates. On OSX, the two modified enzymes both showed higher mobility than the wild-type enzyme. On WU-AX, in contrast, the NS4W-N141Q mutant displayed a lower mobility than the wild-type enzyme, while the G56A-T183A-W185A mutant showed higher mobility. The results clearly demonstrate that both substrate hydrolysis and substrate targeting are key factors for XBS mobility.



1. INTRODUCTION

Fluorescence recovery after photobleaching (FRAP) is a quantitative fluorescence technique that is used to study protein dynamics.¹ Scanning confocal FRAP is performed on a laser scanning microscope. By illuminating a defined region-of-interest (ROI) in the sample with a brief high-intensity laser pulse, the fluorescence is rapidly photobleached. It will recover due to the exchange of bleached molecules inside with unbleached molecules outside this region.² The technique has been used frequently in living cells to investigate the mobility of proteins that are expressed in fusion with green fluorescent protein.^{3,4} However, only very few FRAP studies have investigated the mobility of carbohydrate-active enzymes on their natural substrates. To our knowledge, FRAP studies on carbohydrate-active enzymes have only been conducted to study the movement of β -amylases on the surface of starch gels⁵ and to investigate the mobility of cellulases and their cellulose-binding domains on crystalline cellulose.⁶ In this study, FRAP is used for the first time to study the movement of a xylanase on two of its natural substrates, i.e., water-unextractable arabinoxylan (WU-AX) and insoluble oat spelt xylan (OSX), and to assess

how substrate hydrolysis and substrate binding affect the mobility of the enzyme.

Xylans are important structural polysaccharides in plant cell walls and the second most abundant polysaccharides in Nature. Therefore, xylanases (EC 3.2.1.8), enzymes that can hydrolyze the backbone of xylans internally, are important in the process of biomass breakdown.⁷ Because of their strong impact on the physicochemical properties of xylans, they are also used in several biotechnological applications to improve processing or product quality.^{8–10} Most xylanases belong to glycoside hydrolase family (GH) 10 and 11 (Carbohydrate-Active EnZymes database, www.cazy.org).¹¹ GH11 xylanases, including the *Bacillus subtilis* xylanase A (XBS) examined in this study, mostly consist of a single domain with a typical β -jelly roll structure.¹² It has been demonstrated that, in XBS, substrates can be bound not only in the active site but also at a noncatalytic secondary xylan binding site (SBS) at the surface

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of the enzyme.^{13,14} The importance of the SBS for the activity and binding properties of XBS has previously been assessed by studying enzymes with a modified SBS.¹⁵ Modification of the SBS of XBS has no effect on the activity toward short oligomeric substrates such as xylohexaose because the SBS is located too far from the active site. However, the activity and affinity toward polymeric substrates are greatly affected. This was demonstrated by elimination of several important interactions in the SBS of XBS by the introduction of three alanine residues (G56A-T183A-W185A) which resulted in a dramatic decrease in enzyme activity and affinity.¹⁵ Attempts to increase the substrate binding affinity of the SBS of XBS led to more ambiguous results. N54W-N141Q mutations gave rise to an increased affinity for WU-AX but a decreased affinity toward OSX. Activity, however, was decreased on both substrates.¹⁵

In this study, the importance of substrate hydrolysis and secondary substrate binding for the mobility of XBS on its natural substrates is investigated using FRAP. To determine the impact of substrate hydrolysis for the mobility of XBS, the recovery after photobleaching on WU-AX and OSX of the fluorescently labeled wild-type XBS was compared to that of an inactive mutant. The inactive XBS was created by mutating the catalytic acid/base residue to Ala (E172A), resulting in a catalytically incompetent enzyme.^{14,16} The importance of secondary substrate binding for the mobility of XBS was assessed by using enzymes with a modified SBS, namely, the mutants G56A-T183A-W185A and N54W-N141Q. The behavior of these two mutants was compared with that of the wild-type XBS in FRAP experiments on WU-AX and OSX. The results presented here clearly show that both substrate hydrolysis and substrate targeting are key factors for xylanase mobility.

2. MATERIALS AND METHODS

2.1. Materials. All chemicals, solvents, and reagents were purchased from Sigma-Aldrich (Bornem, Belgium) and were of analytical grade unless specified otherwise. WU-AX isolated from wheat flour and Xylazyme AX tablets were from Megazyme (Bray, Ireland). Xylans from oat spelts and oligonucleotide primers were from Sigma-Aldrich. The insoluble fraction of oat spelt xylan was obtained by removing the soluble component from the starting material. First, an extract was made of 1.0 mg of xylan/20 mL of water by shaking the solution for 15 min at 4 °C. After centrifugation (1500 × g, 10 min), the residue was boiled for 30 min in 20 mL of water/mg of starting material. Following a new centrifugation step (11000 × g, 30 min), the remaining soluble components were removed from the residue by shaking in 20 mL of water/mg of starting material for 15 min at room temperature. After centrifugation (11000 × g, 30 min), the insoluble fraction was lyophilized.

2.2. Substrate Immobilization. Surfaces containing immobilized substrate were prepared by evaporation of a 100 μL drop containing 7.5 mg/mL suspended WU-AX or insoluble OSX on a borosilicate glass #1 microscope coverslip (Knittel Glass, Braunschweig, Germany). The thickness of the substrate layers attained this way was approximately 50 μm for both substrate surfaces.

2.3. Characterization of WU-AX and OSX. The composition of the WU-AX and OSX used for immobilization was assessed by hydrolysis of the materials and analysis of the noncellulosic monosaccharide content with gas chromatography following derivatization as described elsewhere.¹⁷

2.4. Recombinant Production and Purification of Xylanases. The recombinant production and purification of wild-type XBS, N54W-N141Q, and G56A-T183A-W185A have been described elsewhere.¹⁵ The catalytically inactive E172A mutant was constructed with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the pEXP5-CT-xynA construct as a template and 5'-CCAAGTCATGGCGACAGCTG-GATATCAAAGTAGTG/5'-CACTACTTGATATCCAGCT-GTCGCCATGACTTGG as a primer pair. To label XBS and its mutant variants with a fluorescent dye, a sole cysteine residue was introduced in the same way to replace a serine residue in the α-helix of the enzyme (S155C) by making use of 5'-GAACGCATGGAAGTGCCATGGAATGAATC/5'-GATTCAATTCCA-TGGCACTTCATGCGTTC as a primer pair. The enzymes were expressed in *Escherichia coli* BL 21 (DE3) pLysS and purified as described elsewhere.¹⁵ Enzyme purity was evaluated using SDS-PAGE and silver staining performed on a PhastSystem Unit (GE Healthcare, Uppsala, Sweden). Protein concentrations of the purified xylanases were determined on the basis of the extinction at 280 nm measured with a NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and using molar extinction coefficients calculated from the amino acid sequences with the ProtParam tool (<http://expasy.org/tools/protparam.html>).

2.5. Labeling of XBS. The enzymes were labeled by making use of the Alexa Fluor 488 C₅ maleimide dye (Invitrogen, Carlsbad, CA). The 100 μL reaction mixture contained 100 μM enzyme, 1.00 mM tris(2-carboxyethyl)phosphine (TCEP) reducing agent, and 1.00 mM Alexa Fluor 488 C₅ maleimide in sodium HEPES 50 mM pH 7.0 buffer. This reaction mixture was incubated in the dark for 120 min at room temperature. To remove the residual free dye and TCEP from the labeled protein, samples were washed out using Amicon Ultracell-10 filters (Millipore, Billerica, MA) and extensive rinsing with sodium HEPES buffer (50 mM, pH 7.0). By measuring the extinction at 280 and 493 nm, with the Nanodrop-100 Spectrophotometer, the molar ratio of dye over xylanase was calculated as described by the manufacturer.

2.6. Xylanase Activity. After 10 min of preincubation at 40 °C, a Xylazyme AX tablet was added to 1.0 mL of an appropriate enzyme dilution. Enzyme dilutions were prepared in a McIlvaine buffer (100 mM citric acid + 200 mM sodium phosphate, pH 6.0) containing 0.50 mg/mL bovine serum albumin. The reaction was terminated after 60 min of incubation at 40 °C by addition of 10.0 mL of Tris solution (1.0% w/v), vigorous vortex-mixing, and immediate filtration. The extinction of the filtrate at 590 nm was measured. Results are expressed relative to the activity of the wild-type XBS, which was set at 100%.

2.7. FRAP Experiments. FRAP experiments were performed on a LSM510/ConfoCor2 system (Carl Zeiss, Jena, Germany). The 488 nm line of the argon-ion laser was used as excitation light at high intensity (~1.6 mW, front objective aperture) for photo-bleaching and at low intensity (32 μW) to monitor sample fluorescence. A C-Apochromat 40×/1.2 W Korr/0.13–0.17 water immersion objective focused the excitation light in the sample. Emitted fluorescence light was filtered through a 500–550 nm band-pass filter, was passed through a 1 Airy unit confocal pinhole, and was subsequently detected on a photomultiplier tube.

Substrate surfaces were covered with 100 μL of 0.50 μM enzyme in 50 mM sodium HEPES buffer (pH 7.0). Before the start of FRAP experiments, the samples were incubated for at least 1 h to reach steady-state condition. Measurements were done at room temperature (20–22 °C). A circular ROI with a

$2.5\ \mu\text{m}$ radius (ROI1) was chosen for bleaching unless specified otherwise. A second equivalent ROI (ROI2) located nearby was monitored as a control. The average fluorescence in ROI1 was corrected with the average fluorescence in ROI2 and normalized to get a value of 1 before bleaching and of zero immediately after the bleach pulse. The fluorescence intensity was only measured each second, requiring 165 ms to scan the entire ROI, to avoid bleaching-while-acquisitioning.¹⁸ Five scans were taken before the sample was bleached with a short pulse ($\sim 1\ \text{s}$) at high laser intensity. Because the bleaching time was short compared to the time scale of the FRAP experiments, the phenomenon of recovery-during-bleaching was neglectable.¹⁹ The variability on a single FRAP measurement was high, with a typical 15% deviation between the normalized values of single FRAP curves at a certain time point. In order to obtain reproducible results, 10–15 single recoveries were measured on each sample and an average recovery curve was calculated. Standard deviation values are shown as error bars on the FRAP curves. To test the day-to-day reproducibility, average FRAP curves resulting from 10–15 measurements were measured on three separate days. This resulted in a typical error of only 3% on values at each time point, demonstrating good day-to-day reproducibility.

2.8. Data Analysis. Analysis of FRAP curves was performed with a full reaction–diffusion model,²⁰ a standard diffusion model,^{1,21} and a reaction-dominant model.²⁰ As discussed in the Appendix (see the Supporting Information), the standard diffusion model was deemed most appropriate to fit the FRAP curves in this study. Therefore, all derived parameters result from data fits with the latter model.

Standard Diffusion Model. FRAP curves were fitted to a simple diffusion model^{1,21} in Origin 7.0 (OriginLab, Northampton, MA):

$$\text{frap}(t) = \exp\left(\frac{-\tau_D}{2t}\right) \times \left[I_0\left(\frac{\tau_D}{2t}\right) + I_1\left(\frac{\tau_D}{2t}\right) \right] \times (1 - F_{\text{imm}}) \quad (1)$$

The factor $(1 - F_{\text{imm}})$ was incorporated to account for a possible immobile fraction (F_{imm}). τ_D is the characteristic diffusion time of the labeled enzyme through the bleach spot. I_α is a modified Bessel function of the first kind with order α .

When the reaction process is much faster than diffusion, this simplified case of the full reaction–diffusion model can be used to estimate an effective diffusion constant (D_{eff}).²⁰ D_{eff} was calculated from τ_D and the radius of the bleach spot (ω) with eq 2. The ratio of the pseudo-on rate constant over the off rate constant ($k_{\text{on}}^*/k_{\text{off}}$) was derived from eq 3, with $k_{\text{on}}^* = k_{\text{on}} \times [\text{free binding sites}]$, and D_f is the diffusion constant of the free enzyme. D_f of XBS was estimated at $116\ \mu\text{m}^2/\text{s}$ for XBS with the Stokes–Einstein law.³

$$\tau_D = \frac{\omega}{D_{\text{eff}}} \quad (2)$$

$$D_{\text{eff}} = \frac{D_f}{1 + \frac{k_{\text{on}}^*}{k_{\text{off}}}} \quad (3)$$

3. RESULTS AND DISCUSSION

3.1. Production and Characterization of Labeled Enzymes and Substrate Surfaces.

The wild-type XBS used contains no

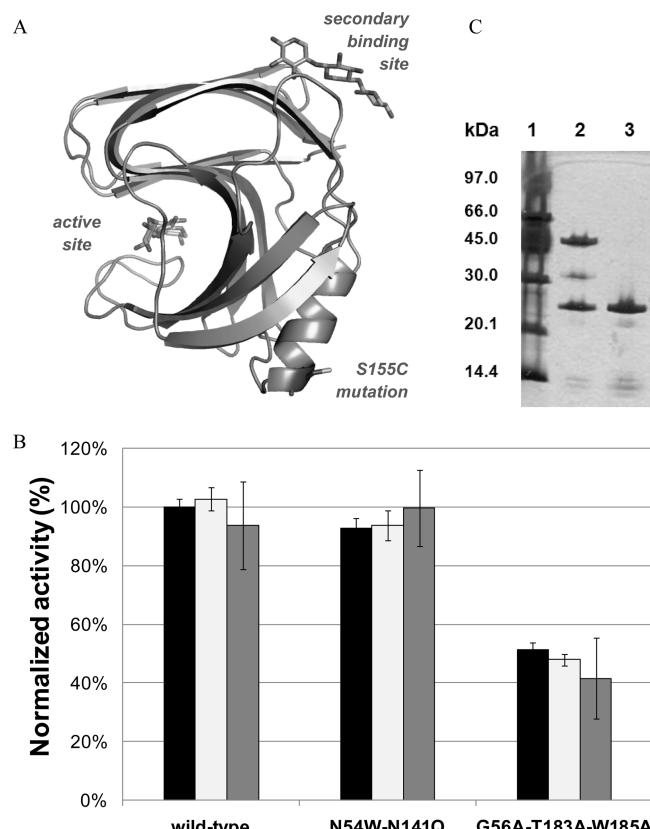


Figure 1. Enzyme labeling. (A) Location of the S155C mutation, incorporated for enzyme labeling, in the structure of XBS. The figure was drawn using PyMOL (DeLano Scientific; <http://pymol.sourceforge.net/>) based on PDB 2QZ3.¹⁴ (B) Xylanase activity measurements of XBS and its mutants, with (white bar) and without (black bar) the S155C mutation and after labeling with Alexa488 (gray bar), clearly show no loss of activity upon incorporation of S155C and upon labeling. All values are expressed relative to the activity of wild-type XBS, which was set at 100%. Error bars represent standard deviations. (C) S155C mutants form cysteine bridge mediated dimers as demonstrated by SDS-PAGE. Lane 1, marker proteins; lane 2, purified XBS_S155C; lane 3, XBS_S155C with addition of 10 mM dithiothreitol.

cysteine residues. Introduction of a sole cysteine residue thus provides control over the position where a thiol-reactive label can be attached and makes it easier to obtain a 1:1 labeling. We mutated Ser155 located in the α -helix of the GH11 xylanase to a cysteine residue (S155C) (Figure 1A). It is reasonable to assume that attachment of a fluorescent group at this position will not impact substrate binding in the active site, on the SBS or at both of these sites simultaneously. The S155C mutation itself appeared to have no effect on the activity of the XBS (Figure 1B), but it did give rise to dimer formation as verified by SDS-PAGE under reducing and nonreducing conditions (Figure 1C). To break up the dimers, the reducing agent TCEP was used. Since TCEP does not contain a thiol group, it is compatible with the maleimide dye that was used for labeling and therefore there was no need to remove the latter from the reaction mixture before adding the dye. For all labeled protein samples, approximately 1 mol of Alexa Fluor 488 C₅ maleimide dye was incorporated per mole of xylanase. The labeled and unlabeled enzymes displayed comparable activities (Figure 1B). As also previously demonstrated,¹⁵ the N54W-N141Q mutant displays an only slightly lower activity on the Xylazyme AX substrate compared

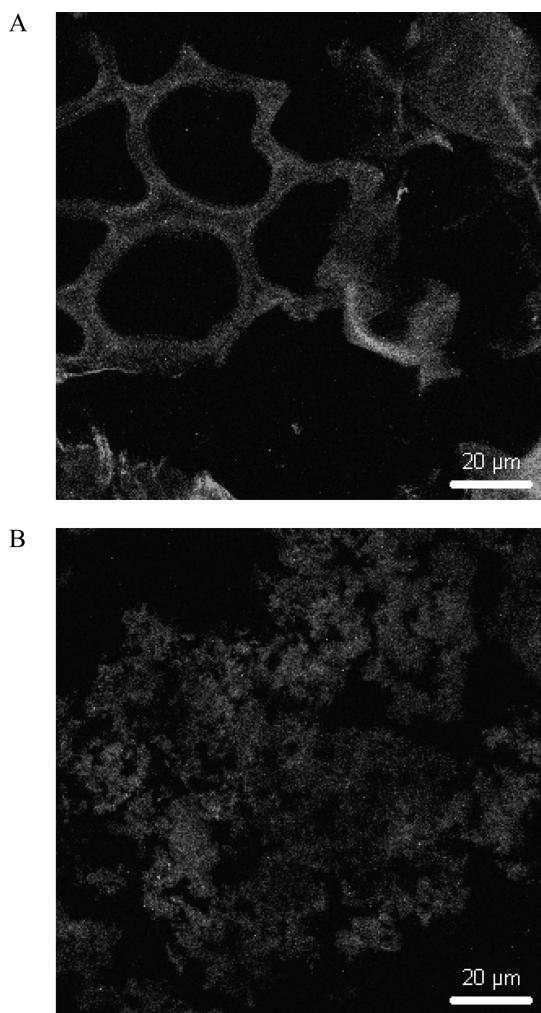


Figure 2. Visualization of substrates. Confocal fluorescence microscopy images of WU-AX (A) and OSX (B) incubated with Alexa488-labeled XBS. The WU-AX contains remnants of aleurone cells.

to the activity of wild-type XBS, while the activity of the G56A-T183A-W185A dropped to half. The E172A mutation indeed resulted in an inactive enzyme.

The wheat flour WU-AX had an estimated arabinoxylan (AX) content of 60% with an average arabinose over xylose ratio of 0.60. The water-unextractability of such AX is caused by inter-molecular cross-linking and interaction with other cell wall components.²² When incubated with fluorescently labeled XBS and visualized with a confocal fluorescence microscope, the substrate appears to be rather heterogeneous (Figure 2A). The majority of the labeled enzymes was located in the cell walls of structures that appeared to be remnants of the aleurone layer of wheat kernels, since these broken or damaged cells had dimensions and cell wall thicknesses similar to those described in the literature for aleurone cells.²³ Therefore, in all FRAP experiments on WU-AX, ROIs were chosen inside these aleurone cell walls. OSX contained around 80% AX with an average arabinose over xylose ratio of 0.08. Partial alignment of the chains, as a result of the low degree of substitution of the AX chain, is thought to be the cause of the insoluble nature of the substrate.²² This substrate appeared to be much more homogeneous (Figure 2B), and therefore, ROIs for FRAP experiments were easier to find. The

images obtained here with confocal fluorescence microscopy suggest that the labeled XBS could be an excellent probe to target AX in cell walls as an alternative to other suggested methods such as the immunolabeling of AX^{24,25} or of xylanases²⁶ or carbohydrate-binding modules (CBMs)²⁷ used as AX probes.

3.2. FRAP Optimization, Data Fitting, and Mobility of the Wild-Type XBS. To optimize the starting conditions of the FRAP experiments, different enzyme concentrations were tested in a 0.10–1.0 μM range. Use of low enzyme concentration made it difficult to visualize the substrate. In the tested concentration range, the FRAP on WU-AX was independent of the enzyme concentration (Figure 3A). Recovery on OSX, on the contrary, was influenced by variation of enzyme concentration, especially at the higher end of the concentration range (Figure 3B). Here, higher enzyme concentration resulted in a faster recovery. A possible explanation for this discrepancy could be that WU-AX and OSX possess a different number of potential binding sites. Since almost all labeled XBS is concentrated nearby the substrate, the level of enzymes used in the experiments probably remains far below the number of binding sites on WU-AX. On the contrary, the highest enzyme concentration might begin to saturate OSX. As a lot of binding sites are occupied, the labeled molecules move further between two binding events, thereby causing a faster recovery of fluorescence. A possible reason for why less binding sites seem to be available on OSX could be that the enzyme cannot reach some regions of the OSX chain because large parts of the chains are aligned with one another. WU-AX, on the contrary, has a much more accessible structure.²² On the basis of the experiments with different enzyme concentrations, a concentration of 0.50 μM seemed suitable for monitoring recovery on both WU-AX and OSX and this concentration was therefore used for all further measurements.

A second parameter that was varied is the bleach spot size. In our FRAP experiment, the recovery rate depended strongly on the size of the bleach spot both for measurements on WU-AX and on OSX. Figure 3C and D shows FRAP of wild-type XBS on WU-AX and OSX with two different spot sizes. Recovery in a bleach spot with a 1.5 μm radius was significantly faster than recovery in a bleach spot of 2.5 μm radius. As described by Sprague and McNally (2005),² this test is a simple way to investigate whether the present FRAP experiments are characterized by a diffusion-uncoupled or diffusion-coupled regime. When the time for a protein to bind is similar to or smaller than the time the protein needs to diffuse across the bleach spot (diffusion-coupled recovery), the recovery rate depends on the bleach spot size.^{2,20} Hence, under the conditions used in this study, FRAP is diffusion-coupled. This is also determinative for the choice of model for appropriate data fitting. As discussed in the Appendix (see the Supporting Information), a standard diffusion model^{1,21} was chosen to fit all obtained FRAP curves. The insets in Figure 3C and D show the relationship between τ_D , obtained after use of the standard diffusion model, and ω^2 . Although for each graph only two data points can be shown, the inset figures suggest a direct proportionality between τ_D and ω^2 , as to be expected for eq 2, thereby also indicating that the standard diffusion model is suitable to fit the FRAP curves.

Table 1 lists the obtained parameters for FRAP experiments with the wild-type XBS. The values obtained for F_{imm} indicate that there is no immobile enzyme fraction on WU-AX or OSX. This confirms what easily can be seen in the FRAP experiments themselves where a complete recovery of fluorescence is observed. This observation seems to be logical, since an immobile fraction of

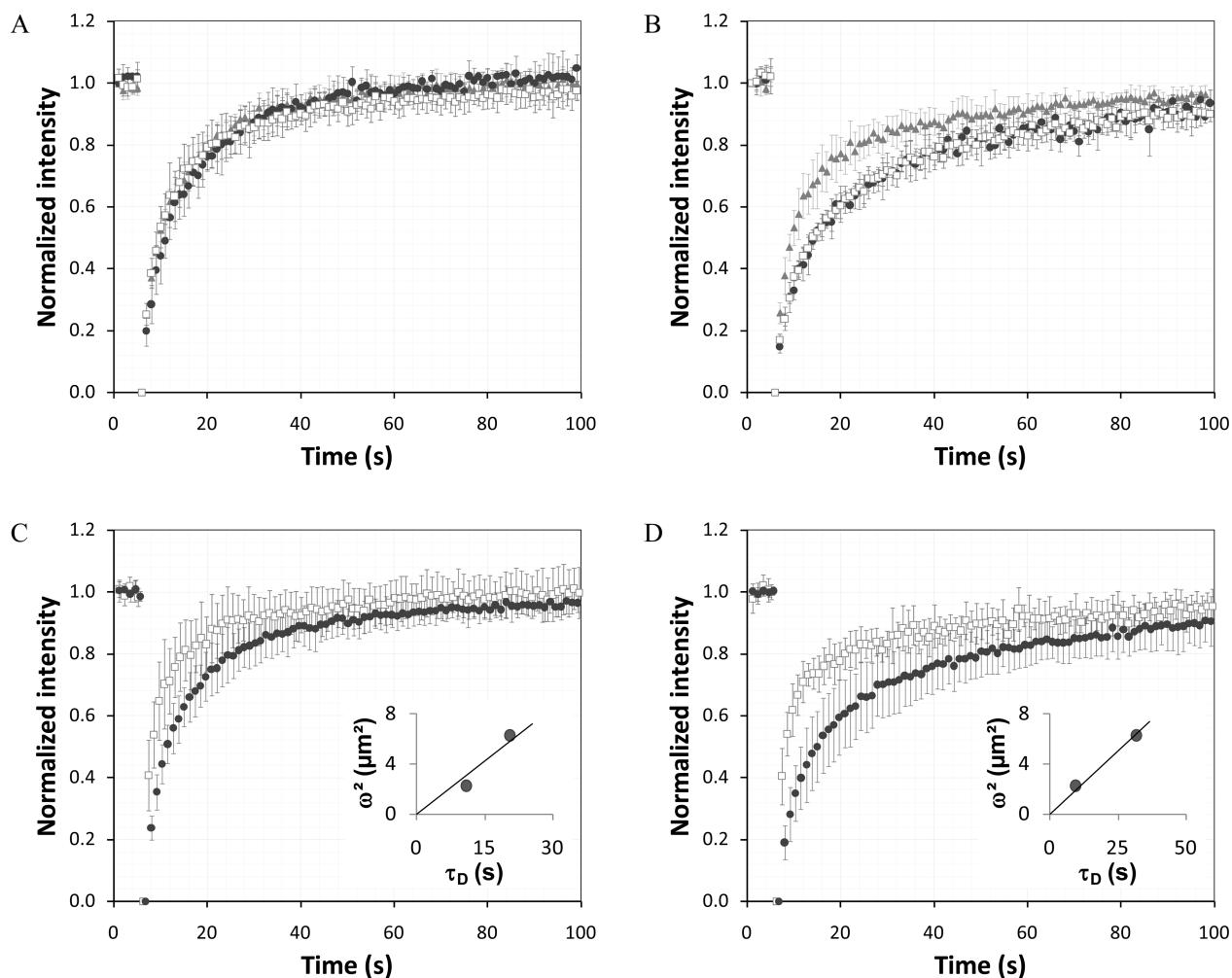


Figure 3. Dependence of FRAP on enzyme concentration and bleach spot size. FRAP curves of wild-type XBS using different enzyme concentrations (\bullet 0.23 μM , \square 0.50 μM , and \blacktriangle 0.93 μM) on WU-AX (A) and OSX (B) and FRAP curves of wild-type XBS using a smaller ($\square \omega = 1.5 \mu\text{m}$) and a larger ($\bullet \omega = 2.5 \mu\text{m}$) bleach spot on WU-AX (C) and on OSX (D). The insets in parts C and D show the relationship between τ_{D} , obtained after fitting the curves with the standard diffusion model, and ω^2 . Error bars show the standard deviation between single FRAP measurements.

Table 1. Parameters Derived from Fitting the Standard Diffusion Model onto FRAP Curves of XBS Wild-Type, G56A-T183A-W185A, and N54W-N141Q on WU-AX and OSX^a

substrate	WU-AX			OSX		
	wild-type	G56A-T183A-W185A	N54W-N141Q	wild-type	G56A-T183A-W185A	N54W-N141Q
Standard Diffusion Model						
adjusted R^2	0.99	0.90	0.99	0.99	0.96	0.99
F_{imm}	-0.02 (± 0.01)	-0.08 (± 0.02)	-0.12 (± 0.04)	0.02 (± 0.02)	0.01 (± 0.02)	-0.02 (± 0.02)
τ_{D} (s)	22 (± 2)	9 (± 1)	69 (± 11)	32 (± 4)	14 (± 1)	28 (± 2)
Derived Parameters						
D_{eff} ($\mu\text{m}^2/\text{s}$)	0.31 (± 0.03)	0.71 (± 0.10)	0.09 (± 0.01)	0.20 (± 0.02)	0.45 (± 0.04)	0.22 (± 0.02)
$k_{\text{on}}^*/k_{\text{off}}$	379 (± 37)	163 (± 8)	1278 (± 93)	586 (± 68)	257 (± 22)	521 (± 43)

^aValues between brackets are standard errors on the mean of the values obtained from fitting single FRAP curves. F_{imm} , immobile fraction; τ_{D} , diffusion time; D_{eff} , effective diffusion constant; $k_{\text{on}}^*/k_{\text{off}}$, pseudo-on rate constant over off rate constant.

enzymes probably cannot play a role in continuous degradation of polymeric substrates. That the recovery is diffusion-coupled implies that the time that an enzyme needs to bind is similar or smaller than the time the enzyme needs to diffuse across the bleached region.

Intuitively, one would expect a short recovery time. However, the time scale of the FRAP experiments is rather long (100 s), indicating a relatively slow recovery of fluorescence. Low k_{off} values can cause slow diffusion-coupled recovery and can be the reason

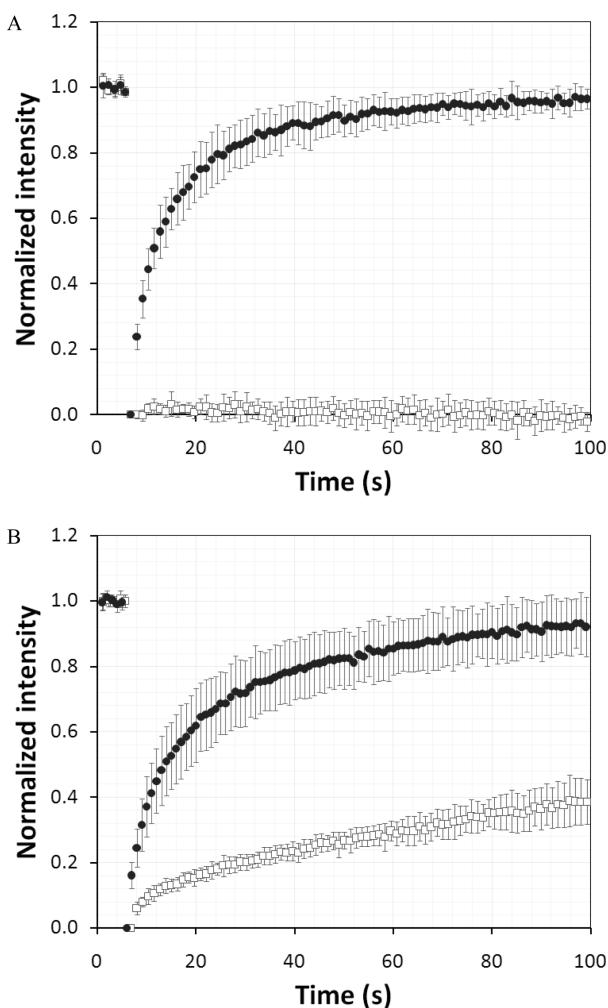


Figure 4. Impact of substrate hydrolysis on FRAP. FRAP measurements of ● wild-type XBS and □ inactive XBS on WU-AX (A) and OSX (B). Error bars show the standard deviation between single FRAP measurements.

that diffusion contributes throughout the long recovery.²⁰ D_{eff} can be calculated on the basis of eq 2 and the estimated τ_D in the standard diffusion model. The hereby obtained D_{eff} values are 0.31 and $0.20 \mu\text{m}^2/\text{s}$ for recovery of labeled wild-type XBS on WU-AX and on OSX, respectively. On the basis of an estimated diffusion constant for free XBS, the ratio $k_{\text{on}}^*/k_{\text{off}}$ can be calculated from this. Table 1 shows the results. The obtained values indicate that almost all the enzyme molecules in our experiment are associated with substrate. Since separate values for k_{on}^* and k_{off} are not deducible from the standard diffusion model and since k_{on}^* depends on the amount of free binding sites, it is impossible to compare the obtained values with literature values. For the same reason, it is also not possible to compare values obtained for the two different substrates. One can only compare different enzymes on the same substrates, as done in the next sections.

To gain more insight into the interaction of XBS with its substrates, a few small tests were conducted. After WU-AX and OSX surfaces had been incubated with labeled XBS for 1 h, the samples were washed with buffer or incubated with an excess nonlabeled wild-type XBS. Washing with buffer did not appear to result in loss of fluorescence. Incubation with nonlabeled enzyme, in contrast, led to a gradual loss of fluorescence on the substrate.

This indicated that, although the enzymes are closely associated with the substrate, they do come loose when the nonlabeled enzymes compete with the labeled ones for binding to the substrate. These results suggest that, once the enzyme is targeted to the substrate, it remains in the neighborhood of the substrate before rebinding at a different site. This reduction of dimensionality in which the enzyme can diffuse freely can greatly enhance enzyme activity.²⁸

3.3. Mobility of the Inactive XBS. To investigate the role of hydrolysis for the mobility of XBS on its natural substrates, the behavior of a catalytically inactive mutant of XBS (E172A) was compared with that of the wild-type XBS. The fluorescently labeled E172A mutant was also incubated on WU-AX and OSX covered slides. Especially on WU-AX, the inactive XBS appeared to have more difficulty to penetrate the substrate. Indeed, in the measurements, only the edges of the aleurone cell wall structure became visible under the fluorescence microscope. The wild-type enzyme can probably open up the cell wall structures by hydrolysis of some AX and can therefore penetrate more easily.²⁶ FRAP experiments with this enzyme revealed large differences in comparison with FRAP of the wild-type XBS (Figure 4). On WU-AX, no fluorescence recovery was observed in the bleached area, showing that the inactive XBS was completely immobile on this substrate (Figure 4A). On OSX, partial recovery of fluorescence was seen in the time scale of the experiment. However, this recovery was very slow when compared to that of the wild-type XBS (Figure 4B). The reason why the inactive XBS recovers slowly on OSX while it remains immobile on WU-AX is unclear. The fact that mobility of the XBS is seriously hampered or even made impossible by absence of substrate hydrolysis indicates that the enzyme binds the substrate tightly. The necessity of hydrolysis for normal enzyme mobility is a possible indication for the presence of a mechanism that actively removes the hydrolyzed substrate out of the active site. Such a mechanism, in which the hydrolyzed substrate is catapulted away from the active site by a hinge movement of the thumb-like loop of XBS, has already been suggested.²⁹ Several other studies can be found where the necessity of hydrolysis for mobility is demonstrated in enzyme–substrate systems. A reduction of enzyme mobility was observed upon inactivation of a β -amylase by alkylation⁵ and of a lipase by attachment of a fluorescent label.³⁰ An inactive cellobiohydrolase mutant was demonstrated to be incapable of sliding along crystalline cellulose, while the active enzyme displayed a processive movement.³¹ Furthermore, a catalytically inactive mutant of a phospholipase resided on the same location on the edge of a phospholipid bilayer during the entire time it was visualized, while the residence time of the active enzyme was much shorter.³²

3.4. Mobility of Enzymes with a Modified SBS. WU-AX FRAP experiments were performed with the labeled wild-type XBS and two mutant enzymes with a modified SBS, namely, mutants G56A-T183A-W185A and N54W-N141Q. The activity and binding properties of these enzymes are exhaustively described elsewhere.¹⁵ In short, the binding of wild-type XBS, G56A-T183A-W185A, and N54W-N141Q to WU-AX is characterized by apparent dissociation constants (K_d) of 8.8, 25, and 5.3 mg/mL, respectively. However, both G56A-T183A-W185A and N54W-N141Q display lower activity on WU-AX than the wild-type enzyme.¹⁵ Figure 5A shows results of the FRAP experiment with these three enzymes on WU-AX. The observed differences between the enzymes are rather large and seem to be well correlated with differences in binding affinity. The G56A-T183A-W185A

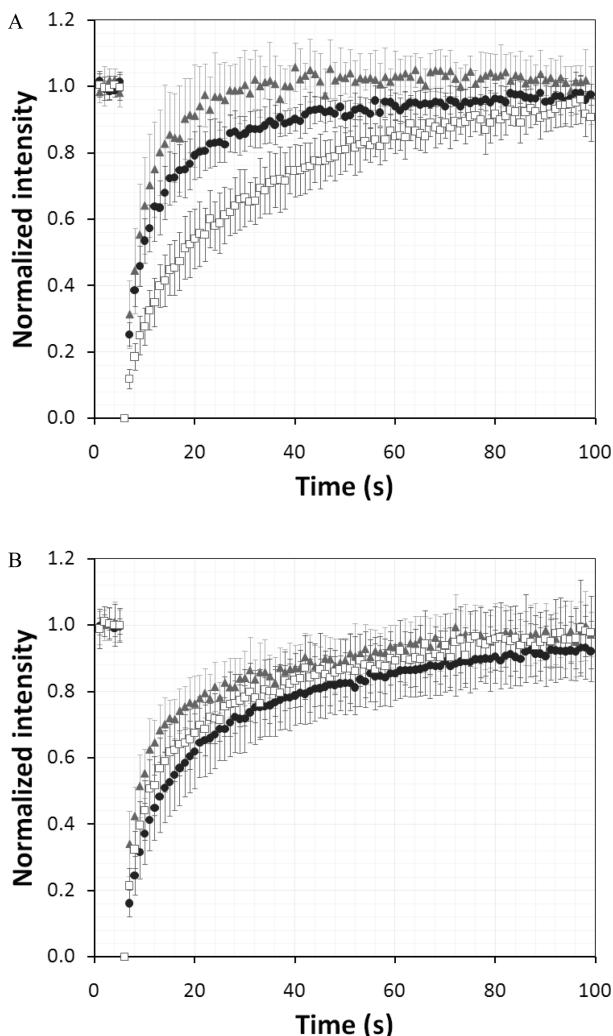


Figure 5. Impact of secondary substrate binding on FRAP. FRAP measurements on WU-AX (A) and OSX (B) of ● wild-type XBS and two modified enzymes in the SBS: ▲ G56A-T183A-W185A and □ NS4W-N141Q. Error bars show the standard deviation between single FRAP measurements.

mutant which showed the lowest affinity toward the WU-AX is the most mobile enzyme. The NS4W-N141Q mutant shows the highest affinity and is the least mobile enzyme on this substrate. These findings are reflected in the τ_D , D_{eff} , and $k_{\text{on}}^*/k_{\text{off}}$ ratio, as summarized in Table 1. A higher D_{eff} means that the enzyme will diffuse further in a certain time and, therefore, reflects higher mobility of the enzyme on this substrate. The $k_{\text{on}}^*/k_{\text{off}}$ ratio provides an indication of the relative importance of enzyme association and dissociation. While for the wild-type enzyme D_{eff} and $k_{\text{on}}^*/k_{\text{off}}$ values of respectively $0.31 \mu\text{m}^2/\text{s}$ and 379 were obtained, these values were $0.71 \mu\text{m}^2/\text{s}$ and 163 for the G56A-T183A-W185A enzyme. On the contrary, NS4W-N141Q mutations gave rise to a much lower D_{eff} and a much higher $k_{\text{on}}^*/k_{\text{off}}$ value of $0.09 \mu\text{m}^2/\text{s}$ and 1278, respectively.

OSX. Figure 5B shows the FRAP curves of measurements of labeled wild-type XBS and the mutants G56A-T183A-W185A and NS4W-N141Q on OSX. Both of these enzymes display a lower binding affinity toward OSX than the wild-type XBS with K_d values of 29 mg/mL (G56A-T183A-W185A) and 2.5 mg/mL (NS4W-N141Q) compared to 0.4 mg/mL for the wild-type. Activity on OSX also decreased for both mutant enzymes.¹⁵ The

recovery curves appear to differ less than those on WU-AX, but as mentioned above, it is very dangerous to compare FRAP measurements between the different substrates. The substrates differ in a lot of properties that are difficult to assess, including their number of free binding sites, structural accessibility, and the presence of nonxylan components. As on WU-AX, G56A-T183A-W185A displays the highest mobility. However, NS4W-N141Q appears to be slightly more mobile on OSX than the wild-type XBS. These findings again correlate well with results of affinity measurements of these enzymes toward OSX.¹⁵ Xylanases with higher affinity toward OSX display lower mobility. The obtained FRAP curves were also fitted with the standard diffusion model. Table 1 lists the obtained parameters. Modification of the SBS of XBS resulted in higher D_{eff} values, namely, $0.45 \mu\text{m}^2/\text{s}$ for G56A-T183A-W185A and $0.22 \mu\text{m}^2/\text{s}$ for NS4W-N141Q in comparison to $0.20 \mu\text{m}^2/\text{s}$ for the wild-type. The $k_{\text{on}}^*/k_{\text{off}}$ ratio was lower: 257 for G56A-T183A-W185A and 521 for NS4W-N141Q in comparison to 586 for the wild-type XBS.

3.5. Relevance of the Present Findings. FRAP experiments clearly demonstrate that weakening of the SBS of XBS by introduction of G56A-T183A-W185A mutations leads to a higher enzyme mobility on both WU-AX and OSX. The higher mobility is probably caused by an impaired ability to target the enzyme toward binding sites on the substrates. Ludwiczek et al.¹³ already demonstrated that $k_{\text{on}}/k_{\text{off}}$ ratios for the active site and the SBS are similar, but that separate k_{on} and k_{off} values are much higher for the SBS than for the active site. The high k_{on} value of the SBS is a clear indication for the major role the SBS plays in initial binding and targeting of the enzyme toward its substrate. Upon elimination of important SBS interaction in the XBS by G56A-T183A-W185A mutations, the k_{on} value of the enzyme decreases, as reflected in the decreased $k_{\text{on}}/k_{\text{off}}$ ratios in Table 1. Once the enzyme dissociates from the substrate, it takes a long time for it to be targeted to a new spot on the substrate. The binding frequency of the enzyme is therefore decreased. This is probably also the cause of the decreased capacity of this enzyme to degrade WU-AX and OSX.¹⁵ The active site will probably be more determinative for the release of the enzyme from its substrate, since it is characterized by the smallest k_{off} .¹³ The experiments with an inactive XBS mutant clearly support this rationale, since the hydrolytic actions of the enzyme appear to be indispensable for its movement. Attempts to enhance the binding power of the SBS using site-directed mutagenesis have previously been shown to be ambiguous.¹⁵ Here, the NS4W-N141Q mutant displays a very large decrease in mobility on the WU-AX substrate which goes hand in hand with its higher affinity toward this substrate. The drastically decreased mobility might jeopardize the enzyme's ability to hydrolyze the substrate efficiently because it stays associated with the same part of the substrate too long. In contrast to the result on WU-AX, the mobility of the NS4W-N141Q mutant on OSX is slightly higher than that of the wild-type XBS. This also corresponds to the outcome of previous binding experiments where the NS4W-N141Q mutant displayed a different behavior toward WU-AX and OSX.¹⁵

4. CONCLUSION

The FRAP experiments presented in this study showed that the wild-type XBS was mobile on its natural substrates and that no enzyme fraction was irreversibly bound to the substrate. It was demonstrated that substrate hydrolysis is a major determinant for

the XBS mobility. A catalytically inactive mutant was immobile on WU-AX, and its mobility on OSX was also greatly impaired. The importance of hydrolysis for the mobility of XBS demonstrated the role of the hydrolysis in substrate release and argues for a mechanism that actively removes the hydrolyzed substrate out of the active site.

In general, modifications in the SBS had a large impact on the mobility of XBS. Besides a major role in hydrolysis of and affinity toward polymeric substrates,¹⁵ the strength of the SBS was also a decisive factor in the movement of the enzyme over its natural substrates. A higher affinity in binding tests was correlated with a lower mobility in FRAP experiments. The SBS probably has an important role in targeting the enzyme toward its substrate. Weakening of the SBS impaired this targeting, thereby leading to a lower binding frequency of the enzyme with its substrate.

■ ASSOCIATED CONTENT

S Supporting Information. An Appendix is available on the choice of the model used to fit the FRAP data described in this manuscript. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ ABBREVIATIONS

AX, arabinoxylans; CBMs, carbohydrate-binding modules; D_{eff} , effective diffusion constant; F_{imm} , immobile fraction; FRAP, fluorescence recovery after photobleaching; GH, glycoside hydrolase family; k_{off} , off rate; k_{on}^* , pseudo-on rate; OSX, insoluble oat spelt xylan; ROI, region of interest; SBS, secondary xylan binding site; TCEP, tris(2-carboxyethyl)phosphine; WU-AX, water-unextractable arabinoxylan; XBS, *Bacillus subtilis* xylanase A; τ_D , diffusion time

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