The family 42 carbohydrate-binding module of family 54 α -L-arabinofuranosidase specifically binds the arabinofuranose side chain of hemicellulose

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α -L-Arabinofuranosidase catalyses the hydrolysis of the α -1,2-, α -1,3-, and α -1,5-L-arabinofuranosidic bonds in L-arabinose-containing hemicelluloses such as arabinoxylan. AkAbf54 (the glycoside hydrolase family 54 α -L-arabinofuranosidase from *Aspergillus kawachii*) consists of two domains, a catalytic and an arabinose-binding domain. The latter has been named AkCBM42 [family 42 CBM (carbohydrate-binding module) of AkAbf54] because homologous domains are classified into CBM family 42. In the complex between AkAbf54 and arabinofuranosyl- α -1,2-xylobiose, the arabinose moiety occupies the binding pocket of AkCBM42, whereas the xylobiose moiety is exposed to the solvent. AkCBM42 was found to facilitate the hydrolysis of insoluble arabinoxylan, because mutants at the arabinose binding site exhibited markedly decreased activity. The results of bind-

ing assays and affinity gel electrophoresis showed that AkCBM42 interacts with arabinose-substituted, but not with unsubstituted, hemicelluloses. Isothermal titration calorimetry and frontal affinity chromatography analyses showed that the association constant of AkCBM42 with the arabinose moiety is approximately 10^3 M^{-1} . These results indicate that AkCBM42 binds the non-reducing-end arabinofuranosidic moiety of hemicellulose. To our knowledge, this is the first example of a CBM that can specifically recognize the side-chain monosaccharides of branched hemicelluloses.

Key words: arabinofuranosidase, arabinose, arabinoxylan, carbohydrate-binding module, glycoside hydrolase, hemicellulose.

INTRODUCTION

The plant cell wall has a composite structure consisting mainly of celluloses, hemicelluloses and lignins. The degradation of cellulose and hemicellulose is catalysed by carbohydrate-active enzymes, such as GHs (glycoside hydrolases), which have been classified into more than 100 families based on amino acid sequence similarity (see the Carbohydrate Active enZYmes server at <http://afmb.cnrs-mrs.fr/CAZY>) [1,2]. GHs are frequently associated with comparatively small non-catalytic CBMs (carbohydrate-binding modules), alongside the GH catalytic domain. CBMs enhance the hydrolysis of insoluble carbohydrates, such as celluloses and hemicelluloses, by binding to them [3–5], as well as keeping the catalytic domain of the enzyme in proximity to the carbohydrates through their sugar-binding activity. CBMs exhibit various types of structures and modes of substrate binding and are presently classified into more than 40 families based on sequence and structural similarity (see the CAZY website, <http://afmb.cnrs-mrs.fr/CAZY>) [6]. A classification of CBMs based on structural and functional similarities has been proposed

in which these protein modules are grouped into three types [7]. Type A CBMs have a flat hydrophobic surface that interacts with adjacent chains on the surface of crystalline cellulose, whereas Type B CBMs have a cleft that accommodates the single glycan chains of poly- or oligo-saccharide ligands. Both Type A and Type B CBMs show relatively high affinity, with association constants greater than 10^5 . In contrast, Type C CBMs have surface pockets and indentations that accommodate oligo-saccharide ligands [8–12], and they exhibit the lectin-like property of binding optimally to mono-, di- or tri-saccharides.

α -L-Arabinofuranosidases (EC 3.2.1.55) catalyse the hydrolysis of the α -1,2-, α -1,3- and α -1,5-L-arabinofuranosidic bonds in L-arabinose-containing hemicelluloses such as arabinoxylan and L-arabinan [13,14]. Arabinoxylan has a β -1,4-linked xylopyranose backbone, and generally contains heterogeneous side-chain substituents such as L-arabinose [15]. The α -L-arabinofuranosidases belong to five GH families: 3, 43, 51, 54 and 62.

Aspergillus kawachii is an industrially important fungus in *shochu* (traditional Japanese liquor) brewing [16]. *A. kawachii*

Abbreviations used: A₁X₂, arabinofuranosyl- α -1,2-xylobiose; A₁X₃, arabinofuranosyl- α -1,2-xylotriose; A₂, arabinobiose; A₃, arabinotriose; A₄, arabinotetraose; AkAbf54, glycoside hydrolase family 54 α -L-arabinofuranosidase from *Aspergillus kawachii* IFO4308; AkCBM42, family 42 carbohydrate-binding module of AkAbf54; CBM, carbohydrate-binding module; FAC, frontal affinity chromatography; H₂PCAbf, glycoside hydrolase family 54 α -L-arabinofuranosidase from *Hypocrea jecorina* PC-3-7; GH, glycoside hydrolase; HPAEC-PAD, high-performance anion-exchange column-pulsed amperometric detection; ITC, isothermal titration calorimetry; mA₁, methyl- α -L-arabinofuranoside; mX₁, methyl- β -D-xylopyranoside; PA, pyridylaminated; pNPA₁, p-nitrophenyl α -L-arabinofuranoside; X₂, xylobiose; X₃, xylotriose.

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The atomic coordinates and structure factors (codes 2D43 and 2D44) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ, U.S.A. (<http://www.rcsb.org/>).

Figure 1 Ribbon diagram of the AkAbf54 E221A mutant in a complex with A_1X_2

The catalytic domain and arabinose-binding domain (AkCBM42) are shown in light grey and dark grey respectively. Two A_1X_2 molecules and N-linked glycoside chain are shown as stick models.

IFO4308 produces various cellulases and hemicellulases [17–20], including two different α -L-arabinofuranosidases, GH51 arabinofuranosidase and AkAbf54 (the GH family 54 α -L-arabinofuranosidase from *A. kawachii*) [21]. Recently, we have determined the crystal structure of AkAbf54 [22,23], and identified the nucleophile and acid/base residues of AkAbf54 as Glu²²¹ and Asp²⁹⁷ respectively. AkAbf54 has an arabinose-binding domain adjacent to its catalytic domain (Figure 1). The arabinose-binding domain is referred to as AkCBM42, because homologous domains have been classified as a new CBM family, CBM42, which has an internal triple repeat sequence, comprises three sub-domains, α (amino acids 348–399), β (amino acids 400–446) and γ (amino acids 447–499). Each sub-domain is assembled with a pseudo-3-fold-axis in the manner of a β -trefoil fold. Although the structural scaffold of CBM42 is similar to that of CBM13 proteins, CBM42 does not contain the GXXXQX(W/Y) motif of CBM13, and the sugar-binding sites of CBM42 are completely different from those of CBM13. In the complex structure of AkAbf54 with arabinose, one arabinofuranose molecule is present in each pocket of the β and γ sub-domains of AkCBM42, but none is present in the α sub-domain. Since the O-1 atoms of both AkCBM42-bound arabinofuranose molecules are exposed to the solvent, AkCBM42 is thought to bind arabinofuranose residues linked to the xylan backbones of arabinoxylans.

CBM42 is thought to be a Type C CBM due to its folding similarity to CBM13 and its postulated binding to insoluble polysaccharides, but its ligand specificity and binding affinity have not yet been determined. In the present study, to clarify the sugar-binding mode of AkCBM42, we structurally analysed the complex of AkAbf54 with arabino-oligosaccharides. We also assayed the binding and activity of the enzyme, as well as performing calorimetric and affinity chromatography analyses, to show that AkCBM42 specifically recognizes a side-chain sugar (arabinofuranoside moiety) of hemicellulose.

MATERIALS AND METHODS

Site-directed mutagenesis

The previously described expression plasmid, pPICZ α C-AkAbf54 [23], was used for site-directed mutagenesis. The mutant enzymes E221A, D435A and D488A were generated using a QuikChange[®] site-directed mutagenesis kit (Stratagene) and the oligonucleotides 5'-C TGG ATC ATG GTC GAT ATG GCG AAC AAC CTC TTC TCT G-3', 5'-G AAG CAG TTC CAT GAG GCT GCT ACT TTC TGT CC-3' and 5'-CG AAG ACG TCG TTT AAT AAT GCT GTT AGC TTT GAG ATT GAG AC-3' respectively. Three double mutants (D435A/D488A, E221A/D435A, and E221A/D488A) and a triple mutant (E221A/D435A/D488A) were also constructed using these primers. All mutations were confirmed by nucleotide sequencing. The plasmids were used to transform *Pichia pastoris* GS115, and the mutant enzymes were expressed and purified as described previously [23].

Sources of sugars

Wheat arabinoxylan (Ara/Xyl, 41:59), rye arabinoxylan (Ara/Xyl, 49:51), oat spelt xylan, birchwood xylan, sugar beet arabinan, and sugar beet debranched α -1,5-arabinan and A_3 (arabinotriose) were purchased from Megazyme. Arabinose, xylose, X_3 (xylotriase) and mX_1 (methyl- β -D-xylopyranoside) were purchased from Wako Pure Chemical Industries. pNPA₁ (*p*-nitrophenyl α -L-arabinofuranoside) was purchased from Sigma-Aldrich. mA_1 (methyl- α -L-arabinofuranoside) was generously given by Dr M. Kitaoka (National Food Research Institute, Ibaraki, Japan).

A_1X_2 [arabinofuranosyl- α -1,2- X_2 (xylobiose)] was prepared by extensively digesting a solution of 0.02 g of wheat arabinoxylan in 1 ml of 50 mM sodium acetate (pH 5.0) with *A. niger* xylanase (0.2 unit) and xylosidase (0.2 unit) (Sigma-Aldrich) at 310 K overnight. The soluble products in the supernatant were fractionated by HPLC on a size-exclusion column (G2000SWXL; Tosoh), and the fractions containing A_1X_2 were determined by RI (refractive index) detection and collected. Arabinose-free wheat arabinoxylan was prepared by extensively digesting a solution of 0.01 g of wheat arabinoxylan in 4 ml of 50 mM sodium acetate (pH 4.0) with 0.2 unit of AkAbf54 at 320 K for 20 h. The solution was centrifuged at 15 000 *g* for 2 min and the pellet was washed twice with 1 ml of 1 M sodium chloride and 50 mM sodium acetate (pH 5.5) to remove AkAbf54.

Enzyme assays

AkAbf54 activity toward a soluble substrate was determined by incubation with 5 mM pNPA₁ at 313 K for 20 min in 100 mM sodium acetate buffer (pH 4.0), and the reaction was stopped by the addition of 0.3 M sodium carbonate. The liberation of *p*-nitrophenol was measured spectrophotometrically at 405 nm. One unit of enzyme was defined as the activity producing 1 μ mol of *p*-nitrophenol per min.

AkAbf54 activity toward an insoluble substrate was measured using 0.5% (w/v) wheat arabinoxylan. Enzyme and substrate were incubated at 313 K for 20 min in 100 mM sodium acetate buffer (pH 4.0), and the reaction was stopped by the addition of 0.3 M sodium hydroxide. The solution was neutralized with HCl and filtrated, and liberation of arabinose was determined by HPAEC-PAD (high-performance anion-exchange column-pulsed amperometric detection), using a Dionex DXc-500 system and a Carbo Pac PA1 anion-exchange column. One unit of enzyme was defined as the activity producing 1 μ mol of arabinose per min.

Binding assay for insoluble hemicellulose

The binding of AkAbf54 protein with the insoluble polysaccharides wheat arabinoxylan, arabinose-free wheat arabinoxylan, oat spelt xylan and birchwood xylan was performed as described in [24]. AkAbf54 protein (20 µg) in 50 mM sodium acetate buffer (pH 5.5) was incubated on ice for 1 h with 2 mg of polysaccharide in a final volume of 100 µl. The solutions were centrifuged at 15 000 *g* for 2 min, the supernatants containing unbound protein were removed, and each pellet was washed twice with 200 µl of 50 mM sodium acetate buffer (pH 5.5) to completely remove unbound protein. Each pellet was boiled for 10 min in 15 µl of 10 % (w/v) SDS, and the samples were subjected to SDS/PAGE on 10 % (w/v) polyacrylamide gels.

Affinity gel electrophoresis

Affinity gel electrophoresis was performed as described previously [24,25], using sugar beet arabinan, sugar beet debranched α-1,5-arabinan, wheat arabinoxylan and rye arabinoxylan as ligands. Continuous native polysaccharide gels consisted of 7.5 % (w/v) acrylamide and 0.1 % polysaccharide in 25 mM Tris/HCl and 250 mM glycine buffer (pH 8.3). Approximately 7–8 µg of AkAbf54 protein and BSA (as a non-interacting negative control) were loaded on to the gels and were subjected to electrophoresis at 20 mA/gel for 1 h at room temperature (25 °C).

ITC (isothermal titration calorimetry)

ITC measurements were carried out at 25 °C by means of standard procedures using a VP-ITC system (MicroCal). The proteins were dialysed extensively against 20 mM sodium acetate buffer (pH 5.5), and the ligands were dissolved in the same buffer to minimize the heat of dilution. During each titration, a protein sample (200–600 µM) stirred at 300 rev./min in a 1.4 ml reaction cell was injected with 25 successive 10 µl aliquots of a ligand (3.5–12.5 mM) at 150 s intervals. The integrated heat effect was analysed by means of non-linear regression using the MicroCal Origin (version 4) program. The fitted data yielded the association constant (K_a) and the enthalpy of binding (ΔH). The number of binding sites on the protein (n) was fixed during the fitting process. Other thermodynamic parameters were calculated using the standard thermodynamic equation ($-RT \ln K_a = \Delta G = \Delta H - T\Delta S$).

FAC (frontal affinity chromatography)

FAC was performed using an automated FAC system (FAC-1; Shimadzu) as described previously [26]. PA (pyridylaminated) oligosaccharides were prepared and labelled using GlycoTAG (TaKaRa). The effective ligand content of immobilized E221A mutant in the column, B_t , was first determined by concentration-dependence analysis and subsequent Woolf–Hofstee-type plots, i.e. $[A]_0(V_f - V_0) = -K_d(V - V_0) - B_t$ [27], where K_d is the dissociation constant, $[A]_0$ and V_f are the initial concentration and elution volume respectively, of pNPA₁, and V_0 is the elution volume of standard sugar, *p*-nitrophenyl α-D-galactopyranoside, which has no affinity for AkCBM42. Using the basic equation of FAC, i.e. $K_d = (V_f - V_0)/B_t - [A]_0$, the K_a (i.e. $1/K_d$) values for PA-glycans were then determined. In the assays employed in this study, $[A]_0$ was 5 nM, which is negligible compared with $1/K_a$. Thus V_f approached the maximum value which is independent of $[A]_0$. We obtained K_a values of AkCBM42 toward various oligosaccharide derivatives using the equation $K_a = (V_f - V_0)/B_t$.

Table 1 Data collection and refinement statistics for AkAbf54 complex structures

Ligand	A ₁ X ₂	A ₃
Data collection statistics		
Wavelength (Å)	1.000	0.978
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters		
<i>a</i> (Å)	39.5	39.5
<i>b</i> (Å)	98.7	98.8
<i>c</i> (Å)	144.0	144.5
Resolution (Å)	50.0–2.3	50.0–2.8
(outer shell)	(2.38–2.3)	(2.9–2.8)
Unique reflections	25 910	14 668
Completeness (%)	83.2 (67.0)	88.6 (87.5)
<i>R</i> _{merge} (%)	0.104 (0.322)	0.160 (0.463)
Mean $\langle I/\sigma(I) \rangle$	12.2 (2.4)	7.5 (2.0)
Refinement statistics		
<i>R</i> / <i>R</i> _{free} (%)	20.3/24.9	19.9/24.9
Average <i>B</i> -factors (Å ²)		
Protein	27.1	30.3
Waters	27.6	23.8
<i>N</i> -Acetylglucosamines	48.9	46.9
β Sub-domain		
Ara1	32.8	35.1
Xyl1	47.7	
Xyl2	42.3	
Ara2		56.1
γ Sub-domain		
Ara1	43.2	38.9
Xyl1	62.4	
Xyl2	54.3	
Ara2		56.2

X-ray crystallography

An E221A mutant crystal was grown under the same conditions as used for wild-type AkAbf54 [23]. An A₁X₂ complex was prepared by soaking E221A crystals in 10 mM A₁X₂ for 30 min, whereas an A₃ complex was prepared by soaking E221A crystals in 10 mM A₃ for 30 min. The datasets were collected at 100 K with a CCD (charge-coupled device) camera on the BL-5A and BL-6A stations at the Photon Factory, High Energy Accelerator Research Organization. Diffraction images were indexed, integrated and scaled with the HKL2000 program suite [28]. The crystal structure of AkAbf54 (PDB code 1WD3) was used as the initial model. During the first stage of crystallographic refinement, the models removed the side chain of the mutated Glu²²¹ residue. Several rounds of refinement and model correction were carried out using programs CNS1.1 [29] and Xtalview [30]. At the final stage of refinement, A₁X₂ or A₂ (arabinobiose) was added to the model according to the $F_o - F_c$ map. Figures were prepared with Raster3D [31], PyMOL (<http://www.pymol.org>) and Xtalview.

RESULTS

Complex structures of AkAbf54 with A₁X₂ and A₃

To determine the structure of the complex between AkAbf54 and A₁X₂ derived from natural xylan, we used the E221A mutant to prevent hydrolysis of the ligand. An AkAbf54 crystal was soaked in 10 mM A₁X₂ for 30 min and diffracted to 2.3 Å (1 Å = 0.1 nm) (Table 1). The structure was refined to an *R*-factor of 20.3 % and an *R*_{free} factor of 24.9 %. Electron density corresponding to soaked A₁X₂ was found in the β and γ sub-domains of AkCBM42, similar to that observed when AkAbf54 was soaked in

Figure 2 Electron density maps of the complex structures with A_1X_2 (A and B) and A_3 (C and D)

$F_o - F_c$ electron density maps (2σ) were constructed prior to incorporation of each ligand into the model structures. Hydrogen bonds are shown as grey dotted lines. A_1X_2 in the β sub-domain pocket (A) and γ sub-domain pocket (B) of AkCBM42, and A_2 in the β sub-domain pocket (C) and γ sub-domain pocket (D) are shown.

arabinose (Figures 1, 2A and 2B). However, we did not observe electron density of A_1X_2 in the substrate-binding pocket of the catalytic domain, probably because of the E221A mutation. Each sugar moiety of A_1X_2 is assigned as Ara1, Xyl1 (xylose linked to arabinose) and Xyl2 (xylose at the reducing end). In each sub-domain of AkCBM42, the arabinose moiety of A_1X_2 (Ara1) occupied the binding pocket, whereas the xylobiose moiety was exposed to the solvent. AkCBM42 does not seem to possess a cleft that can accommodate the xylan backbone around the binding site.

Ara1 is strictly recognized by the β and γ sub-domain pockets in the same manner. Each aspartate residue, Asp⁴³⁵ in the β sub-domain and Asp⁴⁸⁸ in the γ sub-domain, forms two hydrogen bonds with the O-2 and O-3 atoms of Ara1. These hydrogen bonds seem to be most important for the interaction between AkCBM42 and A_1X_2 . Each histidine residue, His⁴¹⁶ in the β sub-domain and His⁴⁶³ in the γ sub-domain, forms a hydrogen bond with the O-5 atom. Moreover, Ara1 is stacked between two tyrosine residues. This mode of recognition of the arabinose moiety of A_1X_2 is similar to that found for the previously reported complex structure with arabinose [22].

In contrast, the electron density of the xylobiose moiety was not clear, probably because of its weak interaction with the protein. Xyl1 does not interact with AkCBM42, whereas Xyl2 exhibits a weak hydrophobic interaction. In addition, the O-1

atom of Xyl2 bound to the γ sub-domain forms a hydrogen bond with Asn⁴⁶⁶, whereas the O-1 atom of Xyl2 bound to the β sub-domain forms a hydrogen bond with Pro¹⁴² of the catalytic domain. Thus the reducing end of the bound A_1X_2 molecule in the β sub-domain is pointed toward the protein body, not toward the solvent area (Figure 1). The area extrapolated from the Xyl-Xyl chain in the β sub-domain was blocked by Pro¹⁴², indicating that a natural arabinoxylan with a longer backbone cannot bind in this manner. It is therefore likely that this portion, which is derived from the backbone of arabinoxylan, was non-specifically bound to AkACBM42 in the complex. In other words, these weak interactions may have been an artefact because we used a short digested oligopeptide.

We also measured the crystallographic data of the E221A mutant soaked with A_3 at a 2.8 Å resolution (Table 1). As in the A_1X_2 complex structure, one A_3 molecule each was bound in the pockets of the β and γ sub-domains (Figures 2C and 2D). The electron density of the arabinose unit at the non-reducing end (Ara1) could be clearly identified, and it exhibited similar hydrogen-bonding and stacking interactions as those observed in the complexes with A_1X_2 and arabinose. The electron density of the middle arabinose unit (Ara2) was slightly observed, but it was not clear. Similar to the Xyl1 unit in the A_1X_2 complex, there was no interaction between Ara2 and AkCBM42. The reducing

Table 2 Specific activities of AkAbf54 and its mutants

	Specific activity (units · mg ⁻¹ · min ⁻¹)			
	Wild-type	D435A	D488A	D435A/D488A
pNPA ₁	31.5	35.3	38.5	27.8
Wheat arabinoxylan	4.1×10^{-2}	8.8×10^{-3}	5.0×10^{-3}	8.1×10^{-4}

end unit of A₃ was completely disordered. The mode of binding of A₃ again indicated that AkCBM42 specifically recognizes one arabinose unit at the non-reducing end. Energetically favoured conformations of the flexible α -1,5-linkage of arabinobiose and the arabinofuranose ring have been identified by NMR molecular modelling [33,34]. Although we could not confidently select the most abundant conformation of A₂ from the crystallographic data, the electron density peaks corresponding to Ara2 were found in the vicinity of the reducing-end arabinose unit of one of those conformers (GG1-North and GT1-South in the β and γ sub-domain pockets respectively; see Supplementary Figure at <http://www.BiochemJ.org/bj399/bj3990503add.htm>).

Hydrolytic activity toward soluble and insoluble substrates

As described above, Asp⁴³⁵ and Asp⁴⁸⁸ interact with the side-chain arabinose of arabinoxylan through bifurcated hydrogen bonds. To evaluate the importance of these residues in arabinose-binding, the mutant proteins D435A, D488A and D435A/D488A were produced, and their catalytic activities toward soluble oligosaccharides and insoluble polysaccharides were compared with that of wild-type AkAbf54 (Table 2).

We found that none of these mutations had an effect on hydrolysis activity toward a soluble substrate, pNPA₁, indicating that AkCBM42 is unrelated to this activity. In contrast, all of these mutants had significantly reduced activities against the insoluble substrate wheat arabinoxylan. Single mutations of the aspartate residues decreased the activity to 12–21 % of that of wild-type enzyme, and the double mutation (D435A/D488A) decreased this activity further (2.3 %). These results indicate that the two binding sites synergistically support the activity toward the insoluble substrate.

HjPCAbf (GH family 54 α -L-arabinofuranosidase from *Hypocrea jecorina* PC-3-7) has been reported to contain a non-catalytic xylan-binding domain in its C-terminal 18 kDa portion, which corresponds to CBM42 [35]. Similarly, proteolysis of this domain with pepsin reduced its activity towards oat spelt xylan to 2.3 % of that of wild-type enzyme.

Binding assay for insoluble arabinoxylan

When we tested the ability of AkCBM42 to bind to insoluble polysaccharide, we found that the E221A mutant bound to wheat arabinoxylan (arabinose content = 41 %), but not to oat spelt or birchwood xylan (arabinose content < 10 %) (Figure 3A). To evaluate the importance of the amount of side-chain arabinose for AkCBM42 binding, we prepared arabinose-free wheat arabinoxylan by treatment with AkAbf54 overnight. As expected, the E221A mutant did not show binding affinity to arabinose-free wheat arabinoxylan (Figure 3B). Compared with the E221A mutant, the double mutants E221A/D435A and E221A/D488A exhibited significantly reduced binding to wheat arabinoxylan (Figure 3C), and we could not detect binding of the triple mutant E221A/D435A/D488A. The presence of arabinose (100 mM) or A₃ (5 mM) inhibited the binding to arabinoxylan, whereas xylose (100 mM) did not (Figure 3D).

Figure 3 Binding to insoluble polysaccharides assessed by SDS/PAGE

The positions of the AkAbf54 are indicated by arrows. Lanes denoted as M contain molecular-mass markers. (A) Binding of the E221A mutant to various insoluble xylans. Lane 1, birchwood xylan; lane 2, oat spelt xylan; lane 3, wheat arabinoxylan. (B) Binding of the E221A mutant to wheat arabinoxylan (lane 1) and arabinose-free wheat arabinoxylan (lane 2). (C) Binding of various AkAbf54 mutants to wheat arabinoxylan. Lane 1, wild-type; lane 2, E221A mutant; lane 3, E221A/D435A mutant; lane 4, E221A/D488A mutant; lane 5, E221A/D435A/D488A mutant. (D) Binding of the E221A mutant to wheat arabinoxylan in the presence of various oligosaccharides. Lane 1, none; lane 2, 100 mM arabinose; lane 3, 5 mM A₃; lane 4, 100 mM xylose.

Affinity gel electrophoresis analysis

The affinity of AkCBM42 to polysaccharide was also evaluated using affinity gel electrophoresis. Wild-type AkAbf54 interacted with arabinan, wheat arabinoxylan and rye arabinoxylan (Figure 4), showing especially high affinity to rye arabinoxylan, which has a high arabinose content (49 %) (Figure 4E). In contrast, AkCBM42 did not interact with debranched arabinan, which does not contain side-chain arabinose (Figure 4C).

Although the D435A mutant exhibited considerable affinity for wheat arabinoxylan and rye arabinoxylan, it was lower than that of wild-type (Figures 4D and 4E). The D488A mutant and D435A/D488A double mutant showed no apparent affinity to wheat arabinoxylan or rye arabinoxylan (Figures 4D and 4E). These findings indicate that Asp⁴⁸⁸ in the γ sub-domain is more important than Asp⁴³⁵ in the β sub-domain for binding to arabinose-substituted hemicellulose, although both aspartate residues contributed to the affinity.

Analysis of ligand specificity by ITC measurement

To investigate the ligand specificity of AkCBM42 in detail, the binding of this protein to sugar ligands was measured at first using ITC. We used proteins with the E221A mutation in order to prevent the generation of heat of association at the active site.

Figure 4 Affinity gel electrophoresis analysis of the interaction with various polysaccharides

Lane M, molecular-mass markers; lane 1, wild-type; lane 2, D435A mutant; lane 3, D488A mutant; lane 4, D435A/D488A mutant; lane S, BSA. (A) No polysaccharide, (B) arabinan, (C) debranched 1,5- α -arabinan, (D) wheat arabinoxylan and (E) rye arabinoxylan.

When we attempted to measure the binding affinity of the E221A mutant to arabinose, it was too weak for accurate quantification ($K_a \sim 10^2 \text{ M}^{-1}$), which may be due to the low abundance of the furanose form in solution [36]. We therefore used a methylated sugar mA_1 , which is expected to exist solely in the furanose form. The E221A mutant exhibited measurable but low affinity to mA_1 ($K_a = 1.6 \times 10^3 \text{ M}^{-1}$) (Figure 5 and Table 3). The protein also exhibited similar affinity ($K_a = 2.7 \times 10^3 \text{ M}^{-1}$) for A_3 . In contrast, the protein did not exhibit detectable affinity to xylo-oligosaccharides mX_1 or X_3 . The double mutants, E221A/D435A and E221A/D488A, exhibited binding affinities with three orders of magnitude, which is not largely different from the E221A

Figure 5 ITC data on binding of 0.6 mM E221A to 12.5 mM mA_1

The upper panel shows raw binding heat, and the lower panel shows the integrated binding heat minus the dilution control heat.

mutant. The E221A/D435A/D488A triple mutant had no detectable affinity to mA_1 or A_3 .

Although the ligand affinity of AkCBM42 was estimated using this method to some extent, we could not perform the measurement with the appropriate protein concentration due to the limit of solubility of the sample, resulting in the not fully-sigmoidal fitting curve (Figure 5). The binding stoichiometry (n value) was fixed to known values (two for E221A mutant and one for double mutants) during the fitting process, because it is

Table 3 Affinity of AkCBM42 to oligosaccharide estimated by ITC

Protein	Ligand	$K_a (\times 10^3 \text{ M}^{-1})$	$\Delta G (\text{kcal} \cdot \text{mol}^{-1})$	$\Delta H (\text{kcal} \cdot \text{mol}^{-1})$	$T\Delta S (\text{kcal} \cdot \text{mol}^{-1})$	n^a	$M_{\text{tot}}^b (\text{mM})$	c
E221A	mA_1	$1.6 (\pm 0.05)^c$	-4.4	$-5.0 (\pm 0.05)$	-0.6	2.0	0.6	1.9
E221A	A_3	$2.7 (\pm 0.2)$	-4.7	$-3.6 (\pm 0.09)$	1.1	2.0	0.4	2.1
E221A	mX_1	ND ^d	ND	ND	ND	ND		
E221A	X_3	ND	ND	ND	ND	ND		
E221A	glucose	ND	ND	ND	ND	ND		
E221A/D435A	mA_1	$8.3 (\pm 0.3)$	-5.4	$-1.0 (\pm 0.01)$	4.4	1.0	0.2	1.7
E221A/D488A	mA_1	$1.3 (\pm 0.02)$	-4.2	$-3.0 (\pm 0.02)$	1.2	1.0	0.3	0.4
E221A/D435A/D488A	mA_1	ND	ND	ND	ND	ND		
E221A/D435A/D488A	A_3	ND	ND	ND	ND	ND		

^a Number of binding sites on the protein. The values were fixed for curve fitting.
^b The total protein concentration in the cell.
^c Values in parentheses are S.D. from the fit.
^d ND, not detected.

Figure 6 FAC analysis using various oligosaccharides

(A) Woolf-Hofstee type plot of the FAC data used to determine the affinity of AkCBM42 for pNPA₁. (B) Elution profiles of various PA-oligosaccharides obtained with the AkCBM42 column.

Table 4 Affinity of AkCBM42 to oligosaccharide determined by FAC

Ligand	$K_a \times 10^3 \text{ M}^{-1}$
pNPA ₁	4.0 (± 0.1) ^a
A ₁ X ₂ -PA	1.0 (± 0.1)
A ₁ X ₃ -PA	4.4 (± 0.2)
A ₂ -PA	3.8 (± 0.2)
A ₃ -PA	4.1 (± 0.1)
A ₄ -PA	3.7 (± 0.2)
X ₂ -PA	ND ^b
X ₃ -PA	ND

^a Values in parentheses are S.D.

^b ND, not detected.

often impossible to derive accurate thermodynamic parameters with such a low c value when the n value is set to one of the fitting parameters [37].

Analysis of ligand specificity by FAC measurement

To accurately determine the binding affinity of AkCBM42 to sugar ligands, we performed FAC measurement. Using this method, we calculated a K_a value of AkCBM42 for pNPA₁ of $4.0 \times 10^3 \text{ M}^{-1}$ (Figure 6A). We subsequently determined the K_a values of AkCBM42 for arabino-oligosaccharide from the relative binding data, based on that of pNPA₁ (Figure 6B and Table 4). The E221A mutant specifically bound to arabino-oligosaccharides, which contain arabinose residues at their non-reducing ends, such as A₁X₂-PA, A₁X₃-PA, A₂-PA, A₃-PA and A₄-PA (where A₄ is arabinotetraose). In contrast, the protein did not exhibit affinity for the X₂-PA, X₃-PA and 49 core oligosaccharides [38]. The K_a values for each of the arabino-oligosaccharides other than A₁X₂-PA were approximately $4.0 \times 10^3 \text{ M}^{-1}$, similar to the values determined using the ITC method. The K_a value of A₁X₂-PA determined by FAC was, however, much lower ($1.0 \times 10^3 \text{ M}^{-1}$) than that of the other arabino-oligosaccharides. This difference may be due to the PA moiety of A₁X₂-PA, in which the xylopyranose ring at the reducing end was open due to pyridylation. The crystal structure complexed with non-labelled A₁X₂ (Figures 1, 2A and 2B) indicates that the PA moiety of A₁X₂-PA can sterically hinder the protein. Since A₁X₃-PA exhibited similar affinity to other arabino-oligosaccharides, such as A₃-PA, the addition of the xylose backbone may have

circumvented the steric hindrance at the PA moiety, but did not enhance the affinity.

DISCUSSION

All of the results presented above show that AkCBM42 has a function not previously reported in other CBMs. With its two mutually similar binding sites, AkCBM42 specifically binds to the arabinose side-chain moiety of arabinoxylan, but not to the xylan backbone. The binding of AkCBM42 to arabinose side chains enhances the arabinofuranosidase activity of AkAbf54 toward insoluble polysaccharides, especially toward arabinose-rich arabinoxylans. Although several CBMs that can recognize the hemicellulose main chain, or a part of it including the side chain, have been described [7,9], to our knowledge no CBM similar to AkCBM42, which specifically recognizes the side-chain moiety of branched polysaccharide, has been reported to date.

ITC and FAC analyses showed that the K_a of both binding sites with an arabinofuranose moiety were approximately 10^3 M^{-1} . This affinity is not surprising, because CBM42 can bind only one sugar unit. These values are apparently lower than the 'baseline' affinity of 10^4 M^{-1} thought to be necessary for CBM function [7], but such low affinity for a single binding site is not rare in lectins. There are a number of examples of multivalent interactions between lectins and carbohydrate ligands that enhance the affinity, called the 'cluster effect' [39]. As to AkCBM42, the mutation at one of the two binding sites significantly decreased the activity. In the case of interactions with insoluble arabinoxylans, therefore, the cluster effect may also be present, and the binding affinity may be in the range sufficiently high for CBM function (e.g. $> 10^4 \text{ M}^{-1}$). Actually, in assaying whether this domain can effectively support hemicellulose degradation, our results strongly indicate that AkCBM42 facilitates the arabinoxylan degradation of AkAbf54.

Although the ITC measurements showed that the binding affinities of the two binding sites in AkCBM42 were not largely different, affinity gel electrophoresis and activity measurements showed that their contributions to the interaction with insoluble polysaccharides and to hydrolysis activity were not equal, with those of the γ sub-domain being higher. This inequality may be due to the different structural environments around these binding sites. The molecular surface around the binding site in the β sub-domain is concave, whereas that in the γ sub-domain is convex (Figure 1). Thus, the latter appears to be more suitable for binding to the arabinofuranose side chain, even

if it is connected to a longer xylan backbone. Alternatively, the difference may be due to the spatial relationships of the binding sites with the active site, in that the binding site in the γ sub-domain is further from the active site than the site in the β sub-domain (47 Å versus 21 Å), with the former located on the opposite side of the active site. The binding site in the γ sub-domain would therefore be able to cover a wider area for anchoring the enzyme at an arabinofuranose site around the 'picking' point, where the active site works.

All known GH54 open reading frames are expected to contain CBM42, because they exhibit high amino acid sequence similarity in their C-terminal regions. In the CAZy database, CBM42 can also be found in the members of the GH43, SAV1043 and SAV1115 families in the genome sequence of *Streptomyces avermitilis*. SAV1043 is expected to have arabinofuranosidase activity, because it shows high sequence identity (58%) with exo-1,5- α -L-arabinofuranosidase from *S. chartreuses* GS901 [40]. In contrast, some arabinofuranosidases are associated with CBMs that can bind the xylan backbone. For example, GH62 arabinofuranosidase from *S. lividans* strain 1326 has CBM13, which is probably responsible for binding to insoluble oat spelt xylan [41]. In addition, CBM35 (X₄ module) of arabinofuranosidase from *Cellvibrio japonicus* (Abf62A) [24] has been found to interact with unsubstituted oat spelt xylan in a calcium-dependent manner, but its binding affinity is not very high ($K_a = 4.1 \times 10^4 \text{ M}^{-1}$).

Due to its ligand specificity, AkCBM42 occupies a unique position in the classification of Boraston et al. [7], in that it can be classified into Fold Family 2 (β -trefoil) and into Type C. The Type C CBMs are reported to bind optimally to mono-, di- or tri-saccharides. As AkCBM42 recognizes only a monosaccharide unit, it may be an extreme example of a Type C CBM. Some CBMs, mostly Type C, are known to exhibit lectin-like properties, as well as having structural similarities to lectin. For example, the β -trefoil fold of CBM13 is classified into the 'Ricin-B-like family' along with a *bona fide* lectin, ricin toxin B-chain [42]. Moreover, CBM6, 32 and 36 are structurally very similar to fucose-specific lectin from *Anguilla anguilla*, especially regarding the location of their metal ion and carbohydrate-binding sites [43]. Some CBMs (i.e. CBM13, 14 and 18) were initially identified as lectins, and they are therefore thought to share similar evolutionary origins. Due to its structural similarity to ricin toxin B-chain, its binding to small sugar units and its multivalency, CBM42 may be one of the most lectin-like CBMs.

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