

1 **An efficient arabinoxylan-debranching  $\alpha$ -L-arabinofuranosidase of family GH62 from *Aspergillus nidulans***  
2 **contains a secondary carbohydrate binding site**

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27  
28 **Abstract:** An  $\alpha$ -L-arabinofuranosidase of GH62 from *Aspergillus nidulans* FGSC A4 (*AnAbf62A*-m2,3) has unusually  
29 high activity towards wheat arabinoxylan (WAX) (67 U/mg;  $k_{\text{cat}} = 178 \text{ s}^{-1}$ ,  $K_{\text{m}} = 4.90 \text{ mg/ml}$ ) and  
30 arabinoxylooligosaccharides (AXOS) with degree of polymerisation (DP) 3–5 (37–80 U/mg), but about 50 times  
31 lower activity for sugar beet arabinan and 4-nitrophenyl- $\alpha$ -L-arabinofuranoside.  $\alpha$ -1,2- and  $\alpha$ -1,3-linked  
32 arabinofuranose is released from mono-, but not from disubstituted xylose in WAX and different AXOS as  
33 demonstrated by NMR and polysaccharide analysis by carbohydrate gel electrophoresis (PACE). Mutants of the  
34 predicted general acid (Glu<sup>188</sup>) and base (Asp<sup>28</sup>) catalysts, and the general acid pK<sub>a</sub> modulator (Asp<sup>136</sup>) lost 1700-, 165-  
35 and 130-fold activity for WAX. WAX, oat spelt xylan, birchwood xylan and barley  $\beta$ -glucan retarded migration of  
36 *AnAbf62A*-m2,3 in affinity electrophoresis (AE) although the two latter are neither substrates nor inhibitors. Trp<sup>23</sup> and  
37 Tyr<sup>44</sup>, situated about 30 Å from the catalytic site as seen in an *AnAbf62A*-m2,3 homology model generated using  
38 *Streptomyces thermoviolaceus* *SthAbf62A* as template, participate in carbohydrate binding. Compared to wild-type,  
39 W23A and W23A/Y44A mutants are less retarded in AE, maintain about 70 % activity towards WAX with  $K_{\text{i}}$  of WAX

40 substrate inhibition increasing 4–7 fold, but lost 77–96 % activity for the AXOS. The Y44A single mutant had less  
41 effect suggesting Trp<sup>23</sup> is a key determinant. *AnAbf62A-m2,3* seems to apply different polysaccharide-dependent  
42 binding modes and Trp<sup>23</sup> and Tyr<sup>44</sup> belong to a putative surface binding site which is situated at a distance of the active  
43 site and has to be occupied to achieve full activity.

44

45 **Keywords:** Glycoside hydrolase family 62, Inverting mechanism, Arabinoxylan, Arabinoxylooligosaccharides, Affinity  
46 gel electrophoresis, Surface binding site

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## 48 **Introduction**

49

50 Plants supply the most abundant biomass on earth and sustainable utilisation of this renewable resource is very  
51 important for society. Plant cell walls are rich in L-arabinofuranose (Araf) found in arabinan main chains, pectin side  
52 chains and as decorations of arabinoxylan (AX), arabinogalactan and gum arabic. Removal of Araf residues constitutes  
53 a bottleneck in plant biomass conversion (Jordan et al. 2012) and efficient  $\alpha$ -L-arabinofuranosidases (ABFs) (EC  
54 3.2.1.55) are needed for various industrial processes such as bioethanol production (Numan and Bhosle 2006).

55 ABFs occur in glycoside hydrolase families GH3, 43, 51, 54 and 62 of the Carbohydrate Active Enzymes database  
56 (CAZy) (Lombard et al. 2014) and are distinguished by the ability to release 1,2- and/or 1,3-linked Araf from singly or  
57 doubly substituted Xylp residues (Van Laere et al. 1999; Sakamoto et al. 2013). Only GH62 contains exclusively ABFs  
58 and it constitutes glycoside hydrolase clan F (GH-F) with GH43 (Lombard et al. 2014) that comprises ABF and several  
59 other specificities. GH62 is predicted to be inverting similar to GH43 (Kellett et al. 1990; McKie et al. 1997; Kimura et  
60 al. 2000) as was here confirmed experimentally by using NMR, which also demonstrated that *AnAbf62A-m2,3* releases  
61  $\alpha$ -1,3-linked three times faster than  $\alpha$ -1,2-linked Araf. Currently 17 GH62 members have been functionally  
62 characterized and kinetic data are reported for nine (Poutanen 1988; Margolles-Clark et al. 1996; Ransom and Walton  
63 1997; Vincent et al. 1997; Lange et al. 2006; De La Mare et al. 2013; Siguier et al. 2014; Maehara et al. 2014; Wang et  
64 al. 2014; Kaur et al. 2014), while substrate specificity was determined for the remaining eight enzymes (Kellett et al.  
65 1990; Kimura et al. 2000; Hashimoto et al. 2011; Sakamoto et al. 2011). The first GH62 crystal structures – five in total  
66 – were published in 2014 (Siguier et al. 2014; Maehara et al. 2014; Wang et al. 2014; Kaur et al. 2014) and share a five-  
67 bladed  $\beta$ -propeller fold catalytic domain with the six GH43 ABF structures of one fungal and five bacterial enzymes  
68 (Nurizzo et al. 2002; Lombard et al. 2014). Single mutants support the role of invariant glutamic acid and aspartic acid  
69 residues as general acid and base catalyst and of another invariant aspartic acid residue as pK<sub>a</sub> modulator of the acid  
70 catalyst (Pitson et al. 1996; Nurizzo et al. 2002; Siguier et al. 2014).

71 The present study concerns *AnAbf62A-m2,3*, one of the two *Aspergillus nidulans* GH62 enzymes available in the  
72 seminal tool box of *Pichia pastoris* transformants encoding *A. nidulans* plant cell wall degrading enzymes (Bauer et al.  
73 2006). *AnAbf62A-m2,3* has no carbohydrate binding module (CBM) therefore its strong binding to different cell wall  
74 polysaccharides motivated establishing a homology model in which Trp<sup>23</sup> and Tyr<sup>44</sup> were tentatively localized to a  
75 surface binding site (SBS). In the light of the number of GH62 sequences in CAZy which is very recently grown by 40  
76 % we here divided GH62 in four phylogenetics subgroups (Supplementary Fig. S1) rather than just two (Siguier et al.  
77 2014).

78

79 **Materials and methods**

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81 Structural modelling and phylogenetic subgrouping

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83 An *AnAbf62A*-m2,3 model obtained using HHpred (Söding et al. 2005) and the structure of *SthAbf62A* from  
84 *Streptomyces thermoviolaceus* (PDB ID 4O8O) as template was judged as “extremely good/very good” (LGscore 5.1  
85 and MaxSub 0.54) by ProQ (Wallner and Elofsson 2003). Alignment with *SthAbf62A* using PyMol 1.3 (Schrödinger,  
86 LLC, New York, NY, USA; also used for rendering structural models) showed similar secondary structural elements  
87 (prediction server PSIPRED (Buchan et al. 2010)) having two *AnAbf62A*-m2,3 outliers (Thr<sup>202</sup> and Asn<sup>287</sup>) in the  
88 Ramachandran plot. The overall rmsd for C $\alpha$  was 0.15 Å.

89 The catalytic domain (cl14647) was identified by Conserved Domain Database (Marchler-Bauer and Lu 2011) in 142  
90 GH62 sequences (May 15 2015) retrieved from CAZy and a multiple alignment (ClustalW default settings within  
91 MEGA 6 (Tamura et al. 2013)) was generated for building a phylogenetic tree using the maximum likelihood algorithm  
92 with MEGA 6 (Tamura et al. 2013). Peptide Pattern Recognition (Busk and Lange 2013) identified unique sequence  
93 motifs for the subgroups with the following parameters (peptide length: 7; number of peptides: 70; cut-off: 10). The  
94 identities were calculated by aid of ClustalW 2.1 (Li et al. 2015).

95

96 Cloning, mutagenesis, expression and purification of *AnAbf62A*-m2,3

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98 *P. pastoris* X-33 transformants (FGSC database accession no. 10088 and 10106; www.fgsc.net) harbouring full-length  
99 *A. nidulans* FGSC A4 ABF (GenBank ID: AN7908.2) were purchased (Fungal Genetics Stock Centre, School of  
100 Biological Sciences, University of Missouri-Kansas City, MO, USA). A 22 residues predicted signal peptide (SignalP  
101 3.0 (Emanuelsson et al. 2007)) was removed using PCR (Expand High Fidelity DNA polymerase; Roche Diagnostics,  
102 Rotkreuz, Switzerland) (for primers see Supplementary Table S1) and a C2A mutation was introduced to avoid  
103 intermolecular disulfide formation. The construct was cloned (using *EcoRI* and *NotI*; New England BioLabs, Ipswich,  
104 MA, USA) in-frame in pPICZ $\alpha$ A (Invitrogen, Carlsbad, CA, USA) with the sequence for the *Saccharomyces cerevisiae*  
105  $\alpha$ -mating factor and a stop codon upstream of a C-terminal His-tag (QuickChange kit; Stratagene, San Diego, CA, USA;  
106 Supplementary Table S1). A pPICZ $\alpha$ A-*AnAbf62A*-m2,3 transformant (*Escherichia coli* DH5 $\alpha$  selected on low salt LB  
107 medium with 25  $\mu$ g/ml zeocin; Novagen, Nottingham, United Kingdom) was sequenced (Eurofins MWG Operon,  
108 Ebersberg, Germany), linearized (*PmeI*; New England BioLabs, Ipswich, MA, USA), transformed into *P. pastoris* X-33  
109 (Micropulser; Bio-Rad, Hercules, CA, USA), and selected (30 °C, 3 d) on yeast peptone dextrose plates with 100  $\mu$ g/ml  
110 zeocin (Invitrogen, Carlsbad, CA, USA). *AnAbf62A*-m2,3 W23A, D28A, Y44A, D136A, E188A, and W23A/Y44A  
111 mutants were made using site-directed mutagenesis (for primers see Supplementary Table S1) according to the  
112 manufacturer’s recommendations (QuickChange kit; Stratagene, San Diego, CA, USA). *P. pastoris* transformants were  
113 grown in shake flasks in buffered glycerol-complex medium (BMGY; 30 °C, 24 h), harvested (3000g, 10 min, 22 °C)  
114 and resuspended to 1/4 of the BMGY culture volume in buffered methanol-complex medium (BMMY; 22 °C, 96 h;  
115 methanol supplemented to 0.5 % (v/v) every 24 h). Supernatants were filtered (0.45  $\mu$ m Durapore membrane filters;  
116 Millipore, Billerica, MA, USA), 10 fold concentrated and buffer-exchanged to 10 mM sodium acetate pH 5.5 (Pellicon  
117 ultra-filtration unit, 10 kDa cut-off filter; Millipore, Billerica, MA, USA), applied (5 ml/min) onto a 15 ml CptoQ

118 column (GE Healthcare, Little Chalfont, United Kingdom) equilibrated with 10 mM sodium acetate pH 5.5 and eluted  
119 by a linear 0–500 mM NaCl gradient (20 CV) (5 ml/min). Fractions containing *AnAbf62A-m2,3* (monitored by SDS-  
120 PAGE) were pooled, concentrated (4000g; Amicon Ultra-15 centrifugal filter units, 10 kDa cut-off; Millipore, Billerica,  
121 MA, USA) and gel filtrated (Hiload 26/60 Superdex G75 column; GE Healthcare, Little Chalfont, United Kingdom) in  
122 10 mM sodium acetate, 0.15 M NaCl, pH 5.5 (0.5 ml/min). Fractions containing *AnAbf62A-m2,3* were pooled,  
123 concentrated and buffer-exchanged to 10 mM HEPES pH 7.5, applied (2 ml/min) to a 6 ml ResourceQ column (GE  
124 Healthcare, Little Chalfont, United Kingdom) in this buffer and eluted by a linear 0–500 mM NaCl gradient (20 CV; 2  
125 ml/min). Pure *AnAbf62A-m2,3* was pooled, concentrated (to 30–970  $\mu$ M), buffer-exchanged to 10 mM sodium acetate  
126 pH 5.5, added sodium azide to 0.02 % and stored at 4 °C. All steps were carried out at 4 °C.

127

#### 128 Protein analyses

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130 *AnAbf62A-m2,3* wild-type and mutants were analysed by SDS-PAGE (4–12 %; Invitrogen). Molecular mass of wild-  
131 type was determined by ESI-MS (LCT Premier mass spectrometer; Waters, Milford, MA, USA). Briefly, *AnAbf62A-*  
132 *m2,3* was exchanged into 2.3 M ammonium acetate (Micro Bio-Spin P-6 size exclusion columns; Bio-Rad, Hercules,  
133 CA, USA), sprayed from nanoES capillaries (ES380; Proxeon, Odense, Denmark) using the parameters; capillary  
134 voltage: 900–1500 V; sample cone voltage: 25 V; source temperature: 30 °C; and cone gas flow: 20 L/h (N<sub>2</sub>) and  
135 spectra were collected in positive ion mode. The instrument was calibrated with 100 mg/ml CsI in 50 % (v/v)  
136 isopropanol. Spectra were processed by smoothing followed by manual deconvolution (MassLynx V4.1 software;  
137 Waters, Milford, MA, USA). Protein concentration was determined by aid of amino acid analysis (Barkholt and Jensen  
138 1989). The melting temperature ( $T_m$ ) was determined by far-UV CD spectroscopy (see Supplementary Fig. S2).  
139 Deglycosylation by endoglycosidase H was attempted under native and denaturing conditions as recommended by the  
140 manufacturer (New England Biolabs, Ipswich, MA, USA).

141

#### 142 Affinity gel electrophoresis

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144 *AnAbf62A-m2,3* and mutants (4  $\mu$ g in sample buffer, 0.25 M Tris base, 0.12 M boric acid, 40 % glycerol, 0.05 %  
145 Bromphenol Blue, pH 8.7) were applied on 12 % (w/v) polyacrylamide gel cast with 0.001–1 % (w/v) low viscosity  
146 wheat AX (WAX-LV) (Megazyme, Wicklow, Ireland), oat spelt xylan, birchwood xylan (both Carl Roth, Karlsruhe,  
147 Germany), larch arabinogalactan, sugar beet L-arabinan (both Megazyme, Wicklow, Ireland), acacia tree gum arabic,  
148 hydroxyethyl cellulose (both Sigma-Aldrich, St. Louis, MI, USA), or barley  $\beta$ -glucan (Novo Industries, Gentofte,  
149 Denmark), and run in 0.25 M Tris base, 0.12 M boric acid, pH 8.7 (4 °C, 50 V, 16 h, XCell SureLock<sup>®</sup> Mini-Cell  
150 system; Invitrogen, Carlsbad, CA, USA) with reference proteins (NativeMark; Invitrogen, Carlsbad, CA, USA) in the  
151 same tank as a control without polysaccharide. Proteins were visualized by Simpleblue SafeStain (Invitrogen, Carlsbad,  
152 CA, USA). WAX-LV was dissolved in water (50 °C) and kept for 1 h. Birchwood and oat spelt xylans were dissolved  
153 in water in a microwave oven, and sugar beet L-arabinan, acacia tree gum arabic, and larch arabinogalactan in water  
154 (RT). Barley  $\beta$ -glucan was wet with a minimum volume 95 % ethanol, suspended in cold water with stirring, heated to

155 boiling with stirring and stirred for 1 h. Hydroxyethyl cellulose was dissolved in 10 mM sodium phosphate pH 6 and  
156 adjusted to pH 8.

157 The relative retardation of migration ( $R_m$ ) by the polysaccharide compared to the control was determined from the  
158 following equation:  $R_m = R_{mi} / R_{mo}$ , where  $R_{mi}$  and  $R_{mo}$  are migration distances of sample relative to reference protein in  
159 the presence and in the absence of polysaccharide, respectively.

160

161 Enzyme activity assays

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163 *4NP-glycosides*: 10 mM 4NPaf, 4-nitrophenyl- $\beta$ -D-xylopyranoside (4NPX) (Sigma-Aldrich, St. Louis, MI, USA) or 4-  
164 nitrophenyl- $\alpha$ -L-arabinopyranoside (4NPAp) (Sigma-Aldrich, St. Louis, MI, USA) in water (20  $\mu$ l) was preincubated  
165 with 125 mM sodium acetate, 0.005 % Triton-X-100, pH 5.5 (20  $\mu$ l; 2 min; 37°C) and added *AnAbf62A-m2,3* (10  $\mu$ l;  
166 12–24  $\mu$ M final concentration). The reaction (10 min; 37 °C) was stopped by 1 M Na<sub>2</sub>CO<sub>3</sub> (200  $\mu$ l) and 4NP quantified  
167 spectrophotometrically at 410 nm (200  $\mu$ l; microtiter plate reader; Bio-Tek Instrument Inc., Winooski, VT, USA) using  
168 4NP (0–0.5 mM) as standard. One activity unit (U) was defined as the amount of enzyme releasing 1  $\mu$ mol/min 4NP.  
169 Kinetic parameters were determined from initial rates of 4NPaf (0.05–4 mM) hydrolysis by *AnAbf62A-m2,3* (0.25–0.5  
170  $\mu$ M; monitored up to 16 min). pH activity optimum was determined using 2.5 mM 4NPaf in 40 mM Britton-Robinson  
171 universal buffers pH 2–10 (Britton and Robinson 1931) and 50 mM sodium acetate pH 5.0–6.0 (37 °C). The  
172 temperature optimum was determined at pH 5.5 for 25–75 °C.

173 *Polysaccharides*: Activity was tested on 0.9 % (w/v) linear L-arabinan (Megazyme Wicklow, Ireland), birchwood  
174 xylan, and barley  $\beta$ -glucan in 50 mM sodium acetate, 0.005 % Triton X-100 pH 5.5 incubated (30 min; 37 °C) with 97  
175  $\mu$ M *AnAbf62A-m2,3* and quantifying reducing sugar by adding 3,5-dinitrosalicylic acid reagent (600  $\mu$ l; 1 % DNS, 0.2  
176 % phenol, 0.05 % NaSO<sub>3</sub>, 1 % NaOH, and 20 % NaK-tartrate), heated (95 °C; 15 min), cooled (on ice; 15 min) and  
177 measuring A<sub>540</sub> (Mohun and Cook 1962) (200  $\mu$ l; microtiter plate reader) using L-arabinose as standard. Specific  
178 activity for 0.9 % WAX-LV, rye AX (Megazyme, Wicklow, Ireland), oat spelt xylan, larch arabinogalactan, sugar beet  
179 L-arabinan, and acacia tree gum arabic was determined for 0.05  $\mu$ M *AnAbf62A-m2,3* in the above buffer (10 min  
180 reaction) and L-arabinose quantified by the lactose/D-galactose (rapid) kit (Megazyme, Wicklow, Ireland) (see below).  
181 One activity unit (U) was defined as the amount of enzyme releasing 1  $\mu$ mol/min arabinose. The effect on hydrolysis of  
182 0.1 % WAX-LV and 0.4 mM or 6 mM 4NPaf by barley  $\beta$ -glucan or birchwood xylan (0.05–0.8 %; 0.1 % with 4NPaf)  
183 was measured assaying released L-arabinose by the lactose/D-galactose kit (see below).

184 Kinetic parameters were determined from initial rates of L-arabinose release (lactose/D-galactose (rapid) kit  
185 (Megazyme, Wicklow, Ireland)) from WAX-LV (0.28–9 mg/ml) and sugar beet L-arabinan (4–90 mg/ml) in the above  
186 buffer (37 °C). Reactions were initiated by adding enzyme (WAX-LV: 0.03–2  $\mu$ M; sugar beet L-arabinan: 1–5  $\mu$ M).  
187 Aliquots (50  $\mu$ l) were removed during 16 min (60 min for catalytic site mutants), added to 1 M Tris-HCl pH 8.6 (200  
188  $\mu$ l) followed by incubation (40 min; RT) with lactose/D-galactose kit solution (880  $\mu$ l) and quantified (200  $\mu$ l; microtiter  
189 plate reader; NADH  $\epsilon_{M,340} = 6300 \text{ M}^{-1} \times \text{cm}^{-1}$ ) using L-arabinose (0–1.75 mM) as standard.  $k_{cat}$ ,  $K_m$ , and  $K_i$  were obtained  
190 (SigmaPlot 9.01; SYSTAT software Inc., San Jose, CA, USA) by fitting either the classical Michaelis Menten  $V = V_{max}$   
191 / (1 + ( $K_m$  / [S]) or the modified equation including a term for uncompetitive substrate inhibition  $V_{i,s} = V_{max} / (1 + ((K_m /$   
192 [S]) + ([S] /  $K_i))$  to initial rate data.  $V$  and  $V_{i,s}$  are reaction rates,  $V_{max}$  maximum rate, [S] substrate concentration, and  $K_i$

193 dissociation constant for inhibited ternary [substrate-enzyme]-substrate complex. Catalytic efficiency ( $k_{cat}/K_m$ ) is  
194 reported for 4NPAf, as  $K_m$  is too high to be determined.

195 Specificity analysis was also done by polysaccharide analysis by carbohydrate gel electrophoresis (PACE) as described  
196 (Goubet et al. 2002) and visualised according to (Bromley et al. 2013). For PACE, WAX was treated with *NpXyn11A*  
197 (Vardakou et al. 2008), *HiAbf43* (McKee et al. 2012) and *AnAbf62A-m2,3* to generate the xylooligosaccharides and  
198 AXOS labelled and used to analyse the specificity of *AnAbf62A-m2,3* essentially as described (McKee et al. 2012).

199 AXOS: Specific activity of *AnAbf62A-m2,3* (final concentration: 0.5  $\mu$ M) was analysed on 53.7 mM (final  
200 concentration)  $A^3X$  [ $\alpha$ -L-Araf-(1 $\rightarrow$ 3)- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)-D-Xylp], 40.7 mM  $A^2XX$  [ $\alpha$ -L-Araf-(1 $\rightarrow$ 2)- $\beta$ -D-Xylp-(1 $\rightarrow$   
201 4)- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)-D- $\beta$ -Xylp], a mixture of 40.7 mM (final concentration)  $A^2XX$  [ $\alpha$ -L-Araf-(1 $\rightarrow$ 2)- $\beta$ -D-Xylp-(1 $\rightarrow$   
202 4)- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)-D- $\beta$ -Xylp] (70 %) plus  $A^3XX$  [ $\alpha$ -L-Araf-(1 $\rightarrow$ 3)- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)-D- $\beta$ -  
203 Xylp] (30 %), a mixture of 32.8 mM (final concentration)  $XA^3XX$  [ $\beta$ -D-Xylp-( $\alpha$ -L-Araf-(1 $\rightarrow$ 3)- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)- $\beta$ -  
204 -D-Xylp-(1 $\rightarrow$ 4)- $\beta$ -D-Xylp (50%) plus  $XA^2XX$  [ $\beta$ -D-Xylp-(1 $\rightarrow$ 4)-[ $\alpha$ -L-Araf-(1 $\rightarrow$ 2)]- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)- $\beta$ -D-Xylp]  
205 (50%), and 32.8 mM (final concentration)  $A^{2+3}XX$  [ $\alpha$ -L-Araf-(1 $\rightarrow$ 2)]-[ $\alpha$ -L-Araf-(1 $\rightarrow$ 3)]- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)- $\beta$ -D-  
206 Xylp-(1 $\rightarrow$ 4)- $\beta$ -D-Xylp] prepared in 33 mM sodium acetate pH 4.5 at 40 °C and released L-arabinose was quantified  
207 using the lactose/D-galactose kit as described previously (McCleary et al. 2015).

208 Relative activities of wild-type (3.7  $\mu$ M), W23A (4.4  $\mu$ M), Y44A (3.3  $\mu$ M) and W23A/Y44A (11  $\mu$ M) were analysed as  
209 above using 2.5 mM  $AX^3$ ,  $XA^2XX+XA^3XX$  and  $A^2XX$  [ $\alpha$ -L-Araf-(1 $\rightarrow$ 2)- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)-D- $\beta$ -  
210 Xylp] (69.5 %),  $XA^3X$  [ $\beta$ -D-Xylp-(1 $\rightarrow$ 4)-[ $\alpha$ -L-Araf-(1 $\rightarrow$ 3)]- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)- $\beta$ -D-Xylp] (19 %) plus  $A^3XX$  [ $\alpha$ -L-  
211 Araf-(1 $\rightarrow$ 3)- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)- $\beta$ -D-Xylp] (11.5 %) (Barry McCleary, in house collection).

212 Action pattern towards  $\alpha$ -1,2- and  $\alpha$ -1,3-Araf decorated Xylp and the stereochemical course were both determined by  
213 NMR. Hydrolysis of 1 mg/ml of  $XA^3XX+XA^2XX$  by *AnAbf62A-m2,3* (0.03 nM),  $A^{2+3}X$  [[ $\alpha$ -L-Araf-(1 $\rightarrow$ 2)]-[ $\alpha$ -L-  
214 Araf-(1 $\rightarrow$ 3)]- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)- $\beta$ -D-Xylp] (by 0.25  $\mu$ M *AnAbf62A-m2,3*), and  $A^{2+3}XX$  [[ $\alpha$ -L-Araf-(1 $\rightarrow$ 2)]-[ $\alpha$ -L-  
215 Araf-(1 $\rightarrow$ 3)]- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)- $\beta$ -D-Xylp-(1 $\rightarrow$ 4)- $\beta$ -D-Xylp] (by 0.25  $\mu$ M *AnAbf62A-m2,3*) in 10 mM sodium  
216 phosphate pH 6 was monitored (800 MHz Bruker Avance II NMR spectrometer equipped with a TCI cryoprobe;  
217 Bruker, Billerica, MA, USA) at 308 K and referenced relative to acetone ( $\delta^1H=2.225$  ppm;  $\delta^{13}C=30.89$  ppm).  $A^{2+3}X$   
218 and  $A^{2+3}XX$  are kind gifts of Maija Tenkanen. For kinetic experiments a series of 1D proton spectra were recorded and  
219 for assignment a series of homo- and heteronuclear 2D spectra were recorded as DQF-COSY, NOESY with 600 ms  
220 mixing time, TOCSY with a spin lock field applied for 60 ms, a multiplicity edited  $^1H$ - $^{13}C$  HSQC and a  $^1H$ - $^{13}C$  HMBC.  
221 The stereochemical course of  $XA^2XX+XA^3XX$  hydrolysis was followed at 308 K by  $^1H$  NMR with single scan 1D  
222 proton experiments of 11.5 s intervals. The first spectrum was recorded 23 s after enzyme addition.

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## 224 Results

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### 226 GH62 phylogenetic subgrouping

227

228 Phylogenetic analysis combined with a peptide pattern search using PPR (Busk and Lange 2013) of 142 GH62  
229 sequences revealed four distinct subfamilies (Supplementary Fig. S1). GH62\_2, the largest subfamily, contains 103 55–



230 100 % identical amino acid sequences and corresponds to the GH62\_2 subfamily defined previously (Siguier et al.  
231 2014). GH62\_1 has 25 39–100 % identical sequences, GH62\_3 and GH62\_4 each have seven 29–100 % and 57–85 %  
232 identical sequences, respectively. *AnAbf62A*-m2,3 belongs to subfamily GH62\_2 (Supplementary Fig. S1). It remains  
233 to be uncovered if these subfamilies and the assigned unique sequence motifs (Supplementary Fig. S3) represent distinct  
234 enzymatic properties. Enzyme kinetics is reported for two GH62\_1 (Couturier et al. 2011; Siguier et al. 2014; Kaur et  
235 al. 2014) and 12 GH62\_2 members (Poutanen 1988; Vincent et al. 1997; Kimura et al. 2000; Tsujibo et al. 2002;  
236 Sakamoto et al. 2011; Hashimoto et al. 2011; De La Mare et al. 2013; Siguier et al. 2014; Maehara et al. 2014; Wang et  
237 al. 2014; Kaur et al. 2014; McCleary et al. 2015), whereas no GH62\_3 member has been characterised and one from  
238 GH62\_4 was shown to degrade oat spelt xylan (Kellett et al. 1990).

239

240 Structural model

241

242 The model of *AnAbf62A*-m2,3 generated based on *SthAbf62A* from *S. thermoviolaceus* (PDB ID 4O8O) of 73 %  
243 sequence identity (Wang et al. 2014) showed a five-bladed  $\beta$ -propeller fold domain. Overlays of arabinose and  
244 xylopentaose from structures of *SthAbf62A* (PDB ID 4O8O) and *Streptomyces coelicolor* *ScAf62A* (PDB ID 3WN2)  
245 (Maehara et al. 2014) complexes (Fig. 1) indicated possible substrate interactions in *AnAbf62A*-m2,3 to involve at least  
246 three main chain binding subsites towards the non-reducing end (+2NR, +3NR, +4NR), one subsite towards the  
247 reducing end (+2R) and subsites –1 and +1 accommodating *Araf* to be cleaved off and the *Xylp* it decorates,  
248 respectively. Equivalent residues at these subsites in *AnAbf62A*-m2,3 and the five GH62 crystal structures are shown  
249 (Fig. 2).

250

251 Purification and physico-chemical characterization

252

253 *AnAbf62A*-m2,3 wild-type, three mutants at the catalytic site and three at the putative SBS were obtained in yields of  
254 150–235 mg/l from *P. pastoris* culture supernatants and migrated in SDS-PAGE as two close bands of apparent  
255 molecular weights 34 and 36 kDa (Supplementary Fig. S4). ESI-MS of *AnAbf62A*-m2,3 wild-type gave  $M_r$  of 33327.3  
256  $\pm$  0.3 and 33633  $\pm$  1 differing by 306 for the lower band, while for the upper and minor band five  $M_r$  values in the range  
257 35434–36067 differed by approximately 162 corresponding to one hexose residue. Mass deviations of 139 Da and  
258 2.4–2.8 kDa from the theoretical  $M_r$  of 33188.5, presumably reflect misprocessing of the signal peptide and / or *O*-  
259 glycosylation, which was not eliminated by endoglycosidase H treatment. *AnAbf62A*-m2,3 forms corresponding to  
260 either of the 34 and 36 kDa bands, were purified in extremely low yield (<1%) by gel filtration (Supplementary Fig.  
261 S5), and found to have the same specific activity towards WAX, therefore *AnAbf62A*-m2,3 wild-type and mutants were  
262 characterised without being subjected to this inefficient purification of each form. The conformational stability of wild-  
263 type and mutants was assessed by aid of CD spectroscopy and  $T_m$  values were determined to 71.53  $\pm$  0.28  $^{\circ}$ C (wild-  
264 type), 69.96  $\pm$  0.19 (D28A), 70.11  $\pm$  0.20 (E188A), 62.48  $\pm$  0.18 (D136A), 60.83  $\pm$  0.22 (W23A), 64.63  $\pm$  0.20 (Y44A),  
265 and 55.41  $\pm$  0.49 (W23A/Y44A) (Supplementary Fig. S2A–G).

266

267 Affinity for polysaccharides

268

269 *AnAbf62A*-m2,3 interacted exceptionally strongly with 0.05 % WAX-LV in AE (Fig. 3A) and got still importantly  
270 retarded by 0.001 % WAX-LV ( $R_m=0.67$ ) (Fig. 3C; Supplementary Table S2), oat spelt xylan ( $R_m=0.73$ ) (Fig. 3D;  
271 Supplementary Table S2) or birchwood xylan ( $R_m=0.80$ ) (Fig. 3E; Supplementary Table S2). *AnAbf62A*-m2,3 thus  
272 recognises the xylan backbone as birchwood xylan has very few (< 1%) or no *Araf* substituents (Kormelink and  
273 Voragen 1993; Li et al. 2000). Two closely migrating bands of the *AnAbf62A*-m2,3 control (Fig. 3B) merged in AE  
274 indicating all *AnAbf62A*-m2,3 forms bind polysaccharides. By contrast 1 % sugar beet L-arabinan ( $R_m=1$ ) (Fig. 3G;  
275 Supplementary Table S2), acacia tree gum Arabic ( $R_m=1$ ) (Fig. 3H), or larch arabinogalactan ( $R_m=1$ ) (Fig. 3I;  
276 Supplementary Table S2) did not retard the enzyme in AE even though they are decorated by *Araf* and L-arabinan is a  
277 substrate (Table 1). Notably, *AnAbf62A*-m2,3 contains no CBM but clearly binds to 0.001 % barley  $\beta$ -glucan ( $R_m=0.9$ )  
278 (Fig. 3F; Supplementary Table S2) and hydroxyethyl cellulose (not shown), which are not substrates. This affinity for  
279  $\beta$ -glucans may be reminiscent to the accommodation of cellotriose at the active site in the *PaAbf62A* structure (Siguier  
280 et al. 2014).

281

282 Substrate specificity and mechanism of action

283

284 *AnAbf62A*-m2,3 degraded WAX-LV with exceptional high activity of 67.42 U/mg (Table 1),  $k_{cat} = 178 \text{ s}^{-1}$  and  $K_m = 2.3$   
285 mg/ml (Table 2, Fig. 4A). WAX-LV exerted uncompetitive substrate inhibition with  $K_i = 2.89 \text{ mg/ml}$  (Table 2, Fig. 4A)  
286 and inhibited hydrolysis of 4NPAf by ~60 % (data not shown). *AnAbf62A*-m2,3 has almost the same high activity on  
287 rye AX and oat spelt xylan (Table 1), but low activity without substrate inhibition for sugar beet L-arabinan of  $k_{cat} =$   
288  $1.03 \text{ s}^{-1}$  and  $K_m = 15.63 \text{ mg/ml}$  (Table 2, Fig. 4A, B). *Araf* substituted larch arabinogalactan and acacia tree gum arabic  
289 were extremely poor substrates and unsubstituted sugar beet linear arabinan was not degraded (Table 1). Birchwood  
290 xylan and barley  $\beta$ -glucan were neither substrates of *AnAbf62A*-m2,3 nor inhibited its hydrolysis of WAX-LV and  
291 4NPAf. *AnAbf62A*-m2,3 showed moderate activity with 4NPAf and optimum at pH 5.5 and 50 °C (Table 1;  
292 Supplementary Fig. S6A–C); its activity towards 4NPAP and 4NPX was 2–3 % compared to 4NPAf (Table 1).

293  $^1\text{H}$  NMR analyses demonstrated that *AnAbf62A*-m2,3 hydrolysed 1,2- and 1,3-*Araf* in  $\text{XA}^2\text{XX}+\text{XA}^3\text{XX}$  (1:1) in singly,  
294 but not from 1,2- / 1,3-*Araf* doubly substituted *Xylp* in  $\text{XA}^{2+3}\text{X}$  and  $\text{XA}^{2+3}\text{XX}$  and 1,3- was released three times faster  
295 than 1,2-linked *Araf* (Table 1, Fig. 5, Supplementary Figs. S7 and S8). Additionally,  $^1\text{H}$ -NMR showed that *AnAbf62A*-  
296 m2,3 liberated  $\beta$ -furanose (assigned anomer resonance: 5.283 ppm) from  $\text{XA}^2\text{XX}+\text{XA}^3\text{XX}$  (Fig. 5, Supplementary Fig.  
297 S7). Due to fast mutarotation, however, the anomeric signal decreased considerably already after 1 min (Fig. 5,  
298 Supplementary Fig. S7). The same specificity was determined by PACE using AXOS and WAX as substrates  
299 (Supplementary Fig. S9). *AnAbf62A*-m2,3 attacked  $\text{A}^3\text{XX}$  and  $\text{XA}^2\text{XX}$ , but not the doubly 1,2- / 1,3-*Araf* substituted  
300 *Xylp* in  $\text{XA}^{2+3}\text{XX}$ . Hydrolysis of WAX by *AnAbf62A*-m2,3 followed by *NpXyn11A*, predominantly released  
301  $\text{XA}^{2+3}\text{XX}$ , xylobiose, xylose and arabinose, confirming the specificity of *AnAbf62A*-m2,3 on the polysaccharide.

302 Finally, alanine mutants of the invariant catalytic site Asp<sup>28</sup>, Glu<sup>188</sup> and Asp<sup>136</sup> retained  $7.7 \times 10^{-3}$ ,  $5.9 \times 10^{-4}$  and  $6.1 \times 10^{-3}$   
303 fold of wild-type activity for WAX-LV (Table 2, Fig. 4C). While D28A showed Michaelis-Menten kinetics on WAX-  
304 LV, D136A complied with the uncompetitive substrate inhibition found for wild-type, but  $K_i$  was doubled (Table 2, Fig.  
305 4C). The activity of the general acid E188A mutant was too low for kinetic analysis. The results agreed with the roles in



306 catalysis of the three residues as general base, general acid catalysts and acid catalyst pK<sub>a</sub> modulator, respectively, also  
307 supported by crystal structures of *UmAbf62A*, *PaAbf62A* (Siguier et al. 2014) and *ScAraf62A* (Maehara et al. 2014).

308

309 Interaction at a putative surface binding site

310

311 In the structural model of *AnAbf62A*-m2,3 Trp<sup>23</sup> and Tyr<sup>44</sup> are situated near the active site cleft, at a distance of about  
312 30 Å from the catalytic site in a shallow cleft that runs perpendicular to the active site cleft, and which is almost a  
313 continuation of this (Fig. 1; 6A, B; Supplementary 3D data). Trp<sup>23</sup> is conserved in 71 % of the 142 GH62 sequences,  
314 which all belong to GH62\_2 and six of seven GH62\_3 sequences. Tyr<sup>44</sup> is seen in 10 (7 %) GH62\_2 sequences and all  
315 10 have Trp<sup>23</sup>. The interaction in AE with WAX-LV, oat spelt xylan, birchwood xylan and barley β-glucan clearly  
316 weakened for W23A and W23A/Y44A, but not for the Y44A mutant that displayed essentially wild-type retardation  
317 (Fig. 3C–E; Supplementary Table S2). While W23A/Y44A retained some binding with the AXs and birchwood xylan  
318 in AE, this is not the case for barley β-glucan (Fig. 3C–F; Supplementary Table S2). Thus substitution of two aromatic  
319 residues at a putative surface binding site (SBS) situated outside of the active site cleft differentially affected  
320 polysaccharide binding specificity of *AnAbf62A*-m2,3.

321 Mutation of Trp<sup>23</sup> and Tyr<sup>44</sup> did not dramatically alter  $k_{cat}$  and  $K_m$  for WAX-LV, sugar beet L-arabinan and 4NPAf  
322 (Table 2, Fig. 4A, D). Remarkably, however,  $K_i$  of WAX-LV substrate inhibition increased 4–7 fold for the three  
323 mutants relative to wild-type (Fig. 4D, Table 2) suggesting significant AX interaction involving Trp<sup>23</sup> and Tyr<sup>44</sup> to be  
324 clearly diminished in the mutants accompanied by modest effect on activity (Table 2, Fig. 3A), which can be interpreted  
325 as an effect of lack of or reduced affinity for WAX at the SBS. Remarkably, depending on the mutant and size of  
326 AXOS (Table 3) only 4–23 % activity was the retained even though Trp<sup>23</sup> and Tyr<sup>44</sup> according to the *AnAbf62A*-m2,3  
327 model (Figs. 1 and 6) are not situated at subsites accommodating AXOS for productive binding.

328

## 329 Discussion

330

331 Knowledge on GH62s is important to provide guidance on ABFs best suited for specific applications. For example  
332 addition of *AnAbf62A*-m2,3 to unhydrolysed oligosaccharides from switchgrass treated with commercial enzymes  
333 efficiently improved the extent of conversion (Bowman et al. 2015). While insights on structure, substrate specificity,  
334 and mechanism of action in a broader sense are gained from sequence based classification of ABFs into GH families  
335 (Lombard et al. 2014), understanding of substrate specificity details and linking of functional diversity with  
336 phylogenetics require experimental studies. Comparison of *A. nidulans AnAbf62A*-m2,3 with other GH62 enzymes  
337 underscored its unusually high activity on both AXs and AXOS and disclosed a putative SBS implicated in activity and  
338 interaction with cell wall polysaccharides.

339

340 Activity and structure/function relationships

341

342 *AnAbf62A*-m2,3 cleaves off 1,2- and 1,3-Araf from mono-substituted Xylp in AXOS and AX and the same specificity  
343 was reported for other GH62\_2 members *StAbf62A* (Wang et al. 2014), *StAbf62A* (Kaur et al. 2014), *Penicillium*  
344 *chrysogenum* AXS5 (Sakamoto et al. 2011), *Penicillium funiculosum* ABF62a–c (De La Mare et al. 2013), *Penicillium*

345 *capsulatum* ABF (Lange et al. 2006), and *StAbf62C* of GH62\_1 (Kaur et al. 2014). The rate of release analysed by <sup>1</sup>H  
346 NMR was three times faster for 1,3- than 1,2-*Araf* probably reflecting that 1,3- and 1,2-linked *Araf* residues bind  
347 productively in opposite directions (Maehara et al. 2014; Wang et al. 2014).

348 *AnAbf62A-m2,3* acts on WAX-LV with 67.42 compared to 0.15–13 U/mg reported for 13 other GH62s (Kellett et al.  
349 1990; Vincent et al. 1997; Kimura et al. 2000; Hashimoto et al. 2011; Sakamoto et al. 2011; Couturier et al. 2011; De  
350 La Mare et al. 2013; Siguier et al. 2014; Maehara et al. 2014; Kaur et al. 2014). *S. thermoviolaceus StAbf62A*,  
351 however, shows ~30 U/mg with WAX-HV (HV = high viscosity) of *Araf:Xylp* ratio of 0.5, which is a superior substrate  
352 to WAX-LV with *Araf:Xylp* of 0.3 (Pitkänen et al. 2009) on which *StAbf62A* shows ~18 U/mg (Wang et al. 2014).  
353 *AnAbf62A-m2,3* has  $k_{cat}$  of 178 s<sup>-1</sup> on WAX-LV (Table 2, Fig. 4A) compared to  $k_{cat}$  = 180 s<sup>-1</sup> of *StAbf62A* determined  
354 with the superior substrate, WAX-HV (Wang et al. 2014). Other GH62s gave much lower  $k_{cat}$  of 0.3–1.5 s<sup>-1</sup> against  
355 WAX-LV and WAX-HV (Vincent et al. 1997; De La Mare et al. 2013; Siguier et al. 2014; Maehara et al. 2014; Kaur et  
356 al. 2014).  $K_m$  of *AnAbf62A-m2,3* is 2.3 mg/ml for WAX-LV (Table 2, Fig. 4A), which is intermediate to  $K_m$  values of 1  
357 mg/ml for *AbfB* from *Streptomyces lividans* (Vincent et al. 1997), *ABF62b* and *ABF62c* from *P. funiculosum* (De La  
358 Mare et al. 2013) and 7–12 mg/ml for *StAbf62A* from *S. thermophilum* (Wang et al. 2014), *ScAraf62A* from *S.*  
359 *coelicolor* (Maehara et al. 2014) and *ABF62a* from *P. funiculosum* (De La Mare et al. 2013). *S. lividans AbfB* contains  
360 a putative CBM, for which the specificity has not been tested without the catalytic domain and it is possible therefore  
361 that the binding of xylan stems from the CBM but it cannot be excluded that the interaction is with the catalytic domain  
362 (Vincent et al. 1997). *ABF62c* from *P. funiculosum* has a cellulose binding CBM13 (De La Mare et al. 2013) perhaps  
363 contributing to substrate binding, while *StAbf62A* has a cellulose binding CBM1 (Wang et al. 2014). *S. thermophilum*  
364 *StAbf62C* has  $K_m$  = 3.7 mg/ml (Kaur et al. 2014) which is similar to *AnAbf62A-m2,3* having  $K_m$  = 4.9 mg/ml (Table 2).  
365 *AnAbf62A-m2,3* and *StAbf62A* are subject to substrate inhibition with  $K_i$  of 2.89 (Table 2, Fig. 4A) and 1.5 mg/ml for  
366 WAX-LV and WAX-HV (Wang et al. 2014), respectively.

367 *AnAbf62A-m2,3* is slightly more active on oat spelt xylan and rye AX than *StAbf62A* (Wang et al. 2014) and neither  
368 *AnAbf62A-m2,3* nor five other GH62s degraded birchwood xylan (Vincent et al. 1997; Tsujibo et al. 2002; Hashimoto  
369 et al. 2011; Sakamoto et al. 2011; Wang et al. 2014).

370 GH62s differ conspicuously in activity level for sugar beet L-arabinan and *AnAbf62A-m2,3* thus has 173- and 3-fold  
371 lower and higher  $k_{cat}$  and  $K_m$ , respectively, than on WAX-LV (Table 2, Fig. 4A,B), whereas *PaAbf62A* and *UmAbf62A*  
372 have  $k_{cat}$  3- and 8-fold higher than *AnAbf62A-m2,3* for sugar beet L-arabinan, but these  $k_{cat}$  values were similar to and  
373 only 3-fold higher, respectively, compared to their values obtained with WAX-LV (Siguier et al. 2014). *StAbf62A*,  
374 however, has a 30-fold lower  $k_{cat}$  of 6 s<sup>-1</sup> for L-arabinan than WAX-HV. The ability to accommodate both AX and  
375 arabinan was reported to involve structural movements upon binding of the xylan main chain in *StAbf62A* (Wang et  
376 al. 2014). *AnAbf62A-m2,3* has 3–4 orders of magnitude lower activity for *Araf* substituted larch arabinogalactan and  
377 acacia tree gum arabic than WAX (Table 1) and did not hydrolyse unsubstituted linear sugar beet arabinan. As for other  
378 GH62s  $\alpha$ -L-1,5 linked *Araf* was not a substrate (Vincent et al. 1997; Tsujibo et al. 2002; Hashimoto et al. 2011; De La  
379 Mare et al. 2013; Kaur et al. 2014). *ScAraf62A* was unable to accommodate L-arabinan at the active site as deduced  
380 both from lack of activity and the crystal structure (Maehara et al. 2014). Apparently substrate interactions differ  
381 between *AnAbf62A-m2,3* and *ScAraf62A* although comparison of the *AnAbf62A-m2,3* model and the *ScAraf62A*  
382 structure did not reveal striking dissimilarities anticipated to result in different ability to act on arabinan. Overall we  
383 conclude that the GH62 family presents important quantitative, but little qualitative variation in substrate specificity.

384

385 Catalytic mechanism

386

387 The present study provides experimental evidence for GH62 of its expected inverting mechanism by the release of  $\beta$ -  
388 furanose from AXOS as monitored by  $^1\text{H}$  NMR, which is in accordance with the known inverting mechanism for GH43  
389 (Pitson et al. 1996) constituting clan GH-F with GH62 (Lombard et al. 2014). The very low residual activities for  
390 WAX-LV of catalytic site mutants D28A (general base); E188A (general acid); and D136A ( $\text{p}K_a$  modulator of the acid  
391 catalyst) confirmed their proposed roles in catalysis. In comparison *StAbf62C* and *ScAraf62A* catalytic acid and base  
392 mutants lost activity completely against WAX-LV and 4NPAf (Maehara et al. 2014; Kaur et al. 2014), as did  
393 *SthAbf62A*, for which, however, a mutant of the acid catalyst  $\text{p}K_a$  modulator retained  $2.1 \times 10^{-5}$  fold of wild-type activity  
394 (Wang et al. 2014). A stabilising effect of the  $\text{p}K_a$  modulator on the catalytic site previously proposed in case of GH43  
395 (Nurizzo et al. 2002) may be reflected in the  $9^\circ\text{C}$  loss in  $T_m$  of *AnAbf62A*-m2,3 D136A (Supplementary Fig. S2E, H).

396

397 Possible importance of the non-reducing and reducing end subsites

398

399 At subsites +2R, +1, +1NR, +2NR and +3NR in GH62 structures the residues vary and no hint to the higher activity of  
400 *AnAbf62A*-m2,3 and *SthAbf62A* towards WAX can be deduced from the structures (Fig. 2). At subsite -1 both  
401 *AnAbf62A*-m2,3 and *SthAbf62A* have tryptophan and threonine that interact with the Araf ( $\text{Trp}^{51}$  and  $\text{Thr}^{43}$ , *AnAbf62A*-  
402 m2,3 numbering), whereas the other enzymes have tyrosine and threonine, respectively (Fig. 2). Because the two former  
403 enzymes *AnAbf62A*-m2,3 and *SthAbf62A* have higher activity for WAX than reported for any other GH62 member, we  
404 speculate that tryptophan at subsite -1 may be associated with their unusually high activity.

405 The level of activity of *AnAbf62A*-m2,3 was 22–48-fold higher for different AXOS than for 4NPAf suggesting that  
406 subsites beyond -1 and +1 are important for a perpendicular orientation of the Xylp ring at subsite +1 positioning Araf  
407 into the subsite -1 pocket (Fig. 2) (Maehara et al. 2014) and offer extra backing for productive accommodation of Araf.  
408 Furthermore, two-fold higher specific activity for  $\text{A}^2\text{XX}+\text{A}^3\text{XX}$  (7:3) and  $\text{XA}^2\text{XX}+\text{XA}^3\text{XX}$  (1:1) compared to  $\text{A}^3\text{X}$   
409 possibly reflects importance of subsite +3NR in productive substrate binding.

410

411 Putative surface binding site

412

413 The substrate inhibition by WAX involved  $\text{Trp}^{23}$  and  $\text{Tyr}^{44}$  as the corresponding alanine mutants were less inhibited by  
414 WAX and also showed improved productive binding (Table 2, Fig. 3A). Thus harmful strain or adverse binding in the  
415 productive complex of WAX-LV and wild-type *AnAbf62A*-m2,3 is relieved by these mutations (Table 2, Fig. 4A).  
416 Although modest changes in  $k_{\text{cat}}/K_m$  (65–104%) for 4NPAf supports retained functional integrity of subsites -1 and +1  
417 remarkably, the activity of W23A, Y44A and W23A/Y44A *AnAbf62A*-m2,3 for different AXOS was only 4–23 % of  
418 wild-type (Table 3),  $\text{A}^3\text{X}$  of DP3 being most affected. Activity improved with DP of both 4 ( $\text{A}^2\text{XX}+\text{XA}^3\text{X}+\text{A}^3\text{XX}$ ) and  
419 5 ( $\text{XA}^2\text{XX}+\text{XA}^3\text{XX}$ ). Apparently occupation also of subsites towards the non-reducing end is needed for effective  
420 productive AXOS interaction (Table 2), altogether suggesting that interaction with distal subsites is significant, as  
421 demonstrated for *StAbf62C* by mutational analysis (Kaur et al. 2014). It may be speculated that carbohydrate binding

422 e.g. by AXOS at a secondary site in *AnAbf62A*-m2,3 involving Trp<sup>23</sup> and Tyr<sup>44</sup> allosterically triggers stimulation of  
423 catalysis as known for SBSs in barley  $\alpha$ -amylase (Oudjeriouat et al. 2003; Nielsen et al. 2012) and *Aspergillus niger*  
424 xylanase (Cuyvers et al. 2011). It is likely that 4NPAf is unable to bind at or has low affinity for the SBS and the  
425 W23A, Y44A and W23A/Y44A mutations therefore do not affect activity towards this substrate. As birchwood xylan  
426 and barley  $\beta$ -glucan interact with *AnAbf62A*-m2,3, but are neither hydrolyzed nor inhibiting activity against WAX, we  
427 propose a polysaccharide binding mode exists distinct from the AX substrate complex and involves an SBS containing  
428 Trp<sup>23</sup> and Tyr<sup>44</sup> situated at a distance of the active site region. This is in agreement with the weakened substrate  
429 inhibition by WAX-LV for the three SBS mutants, and especially the weakened interaction for W23A/Y44A leads us to  
430 suggest that the SBS provides prominent interaction with the polysaccharide in conjunction with the active site.  
431 In conclusion, *AnAbf62A*-m2,3 is the most active WAX-LV and AXOS degrading GH62 member reported to date. AE  
432 showed *AnAbf62A*-m2,3 interacts with the Araf decorated WAX-LV and oat spelt xylan as well as birchwood xylan  
433 and barley  $\beta$ -glucan. In conjunction with mutations of aromatic residues situated ~30 Å from the catalytic site as  
434 guided by a structural model of *AnAbf62A*-m2,3, activity on AXs and AXOS suggests this site is important, whether it  
435 constitutes an SBS or formally would be considered is a distal subsite. Important SBSs are recognised in certain xylan-  
436 degrading enzymes in which the SBSs form shallow clefts that are almost perpendicular to the active site cleft, and most  
437 often have a pair of aromatic residues located in the centre of the SBS cleft (Schmidt et al. 1999; De Vos et al. 2006;  
438 Ludwiczek et al. 2007; Vandermarliere et al. 2008). Trp<sup>23</sup> and Tyr<sup>44</sup> in the *AnAbf62A*-m2,3 model are also located in a  
439 shallow cleft perpendicular to the active site (Fig. 6 and Supplementary 3D data), but in the xylanases the SBSs are  
440 typically found on the other side of the enzyme than the active site (Schmidt et al. 1999; De Vos et al. 2006; Ludwiczek  
441 et al. 2007; Vandermarliere et al. 2008) as opposed to *AnAbf62A*-m2,3 where the shallow SBS cleft is almost a  
442 continuation of the active site cleft.  
443 PACE and NMR specificity analysis showed that singly substituting  $\alpha$ -1,2- and  $\alpha$ -1,3-linked arabinofuranose residues  
444 in WAX-LV and AXOS are hydrolysed by *AnAbf62A*-m2,3. The NMR experiments confirmed release of the  $\beta$ -  
445 arabinofuranose anomer in agreement with the inverting mechanism known for GH43 that forms GH clan-H with  
446 GH62, and further demonstrated that  $\alpha$ -1,3- is released faster than  $\alpha$ -1,2-linked arabinofuranose residues from AXOS.

447

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453

#### 454 **Compliance with Ethical Standards**

455

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459

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462  
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464  
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610 **TABLES**611 Table 1: Specific activities for *AnAbf62A-m2,3*

Substrate	Specific activity (U/mg)
Wheat arabinoxylan	67.42 ± 4.53 (1.00)
Rye arabinoxylan	64.24 ± 1.82 (0.95)
Oat spelt xylan	49.14 ± 1.19 (0.73)
Birchwood xylan	n.d.
Barley β-glucan	n.d.
Sugar beet L-arabinan	1.43 ± 0.14 (0.02)
Linear L-arabinan	n.d.
Larch wood arabinogalactan	0.08 ± 0.01 (0.001)
Acacia tree gum arabic	0.25 ± 0.02 (0.003)
4-nitrophenyl α-L-arabinofuranoside	1.66 ± 0.24 (0.02)
4-nitrophenyl β-D-xylopyranoside	0.03 ± 0.01 (0.0004)
4-nitrophenyl α-L-arabinopyranoside	0.04 ± 0.01 (0.001)
A <sup>3</sup> X	37 ± 1.1 (0.55)
A <sup>2</sup> XX	59 ± 0.5 (0.88)
A <sup>2</sup> XX+A <sup>3</sup> XX (7:3)	80 ± 2.1 (1.19)
XA <sup>2</sup> XX+XA <sup>3</sup> XX (1:1)	80 ± 3.5 (1.19)
A <sup>2+3</sup> XX	n.d.

612 n.d., not detected. Relative values are in parentheses. All experiments were done in triplicates.

613 Table 2: Kinetic parameters for hydrolysis of wheat arabinoxylan, sugar beet L-arabinan and 4-nitrophenyl  $\alpha$ -L-arabinofuranoside by *AnAbf62A*-m2,3 wild-type and  
 614 catalytic site (D28A, D136A and E188A) and putative SBS (W23A, Y44A and W23A/Y44A) mutants.

Wheat arabinoxylan	Wild-type	D28A	D136A	E188A	W23A	Y44A	W23A/Y44A
$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	178 $\pm$ 26 (1.00)	0.64 $\pm$ 0.06 (0.00)	0.63 $\pm$ 0.09 (0.00)	n.d	52.11 $\pm$ 9.25 (0.29)	46.54 $\pm$ 3.16 (0.26)	80.21 $\pm$ 10.53 (0.45)
$K_{\text{m}}$ ( $\text{mg}\times\text{ml}^{-1}$ )	4.90 $\pm$ 0.91 (1.00)	2.62 $\pm$ 0.05 (0.53)	1.57 $\pm$ 0.32 (0.32)	n.d.	2.35 $\pm$ 0.63 (0.48)	1.02 $\pm$ 0.13 (0.21)	6.93 $\pm$ 1.15 (1.41)
$k_{\text{cat}}/K_{\text{m}}$ ( $\text{s}^{-1}\times\text{mM}^{-1}$ )	36.37 (1.00)	0.24 (0.01)	0.40 (0.01)	n.d	22.18 (0.61)	45.76 (1.26)	11.56 (0.32)
Specific activity ( $\text{U}\times\text{mg}^{-1}$ )	67.42 $\pm$ 4.53 (1.00)	0.52 $\pm$ 0.02 (0.01)	0.41 $\pm$ 0.02 (0.01)	0.04 $\pm$ 0.00 (0.00)	48.29 $\pm$ 6.98 (0.72)	45.82 $\pm$ 2.05 (0.68)	50.63 $\pm$ 3.26 (0.75)
$K_{\text{i}}$ ( $\text{mg}\times\text{ml}^{-1}$ )	2.89 $\pm$ 0.58 (1.00)	-	6.0 $\pm$ 1.50 (2.08)	-	16.32 $\pm$ 8.51 (5.64)	11.89 $\pm$ 2.19 (4.11)	19.71 $\pm$ 7.68 (6.82)
Sugar beet L-arabinan	Wild-type	D28A	D136A	E188A	W23A	Y44A	W23A/Y44A
$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	1.03 $\pm$ 0.03 (1.00)	-	-	-	0.73 $\pm$ 0.02 (0.71)	0.96 $\pm$ 0.02 (0.93)	0.81 $\pm$ 0.04 (0.79)
$K_{\text{m}}$ ( $\text{mg}\times\text{ml}^{-1}$ )	15.63 $\pm$ 1.25 (1.00)	-	-	-	20.60 $\pm$ 1.72 (1.32)	12.03 $\pm$ 0.79 (0.77)	33.78 $\pm$ 3.47 (2.16)
$k_{\text{cat}}/K_{\text{m}}$ ( $\text{s}^{-1}\times\text{mM}^{-1}$ )	0.07 (1.00)	-	-	-	0.04 (0.57)	0.08 (1.14)	0.02 (0.29)
Specific activity ( $\text{U}\times\text{mg}^{-1}$ )	1.43 $\pm$ 0.14 (1.00)	-	-	-	1.06 $\pm$ 0.08 (0.74)	0.77 $\pm$ 0.08 (0.54)	1.07 $\pm$ 0.01 (0.75)
4-nitrophenyl $\alpha$ -L-arabinofuranoside	Wild-type	D28A	D136A	E188A	W23A	Y44A	W23A/Y44A
$k_{\text{cat}}/K_{\text{m}}$ ( $\text{s}^{-1}\times\text{mM}^{-1}$ )	0.26 $\pm$ 0.01 (1.00)	-	-	-	0.17 $\pm$ 0.00 (0.65)	0.21 $\pm$ 0.02 (0.81)	0.27 $\pm$ 0.01 (1.04)
Specific activity ( $\text{U}\times\text{mg}^{-1}$ )	1.66 $\pm$ 0.24 (1.00)	-	-	-	1.28 $\pm$ 0.03 (0.77)	2.42 $\pm$ 0.08 (1.46)	1.99 $\pm$ 0.12 (1.20)

615 n.d. - not measured. Relative values are in parentheses. All experiments were in triplicates.

616 Table 3: Relative activities on arabinoxylooligosaccharides for *AnAbf62A*-m2,3 wild-type and mutants of the putative  
 617 SBS

AXOS*	Wild-type	W23A	Y44A	W23A/Y44A
A <sup>3</sup> X	1.00	0.04	0.03	0.04
A <sup>2</sup> XX+XA <sup>3</sup> X+A <sup>3</sup> XX	1.00	0.12	0.10	0.11
XA <sup>2</sup> XX+XA <sup>3</sup> XX	1.00	0.25	0.18	0.23

618 All experiments were in triplicates.

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651 **FIGURE LEGENDS**

652 **Fig. 1** Structural homology model of *AnAbf62A*-m2,3 overlaid with xylopentaose (cyan) from *ScAbf62A* (PDB ID  
653 3WN2) and arabinose (orange) from *UmAbf62A* (PDB ID 4N2R). Subsites are labelled according to McKee et al.  
654 (2012). The catalytic residues are light purple and the putative surface binding site residues in dark purple.

655  
656 **Fig. 2** Subsites and side chains shown to interact with xylooligosaccharides in crystal structures of *SthAbf62A* (PDB ID  
657 4O8O) (pink), *StAbf62C* (PDB ID 4PVI) (brown) *UmAbf62A* (PDB ID 4N2R) (green), *PaAbf62A* (PDB ID 4N2Z)  
658 (salmon) and *ScAbf62A* (PDB ID 3WN2) (yellow). Only side chains that differ from *AnAbf62A*-m2,3 (grey) are  
659 included for the above mentioned. Xylopentaose (cyan) from *ScAbf62A* (PDB ID 3WN2) and arabinose (orange) from  
660 *SthAbf62A* (PDB ID 4O8O) are shown. Numbering refers to *AnAbf62A*-m2,3.

661  
662 **Fig. 3** Affinity gel electrophoresis of *AnAbf62A*-m2,3. A) 17 h run with 0.05 % wheat arabinoxylan, B) control  
663 (without polysaccharide) and with 0.001 % C) wheat arabinoxylan, D) oat spelt xylan, E) birchwood xylan, F) barley  $\beta$ -  
664 glucan, G) sugar beet L-arabinan, H) acacia tree gum arabic and I) larch arabinogalactan. Lane 1: marker; lane 2: wild-  
665 type; lane 3: W23A; lane 4: Y44A; lane 5: W23A/Y44A. The lower vertical line shows the migration of *AnAbfGH62A*-  
666 m2,3 wild-type in the control gel without polysaccharide and the upper one shows a marker used to align the gels.

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668 **Fig. 4** Substrate hydrolysis curves by *AnAbf62A*-m2,3 of A) wheat arabinoxylan, B) sugar beet L-arabinan, C) wheat  
669 arabinoxylan by catalytic site mutants and D) 4-nitrophenyl  $\alpha$ -L-arabinofuranoside. *AnAbf62A*-m2,3 wild-type (●),  
670 W23A (■), Y44A (○), W23A/Y44A (□), D28A (▲) and D136A (▼).

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672 **Fig. 5** Time course of hydrolysis by *AnAbf62A*-m2,3 of AXOS (1:1 molar ratio of  $\beta$ -D-Xylp-(1→4)-[ $\alpha$ -L-Araf-(1→2)]-  
673  $\beta$ -D-Xylp-(1→4)- $\beta$ -D-Xylp-(1→4)- $\beta$ -D-Xylp (A<sup>2</sup>XX) and  $\beta$ -D-Xylp-(1→4)-[ $\alpha$ -L-Araf-(1→3)]- $\beta$ -D-Xylp-(1→4)- $\beta$ -D-  
674 Xylp-(1→4)- $\beta$ -D-Xylp (A<sup>3</sup>XX) by *AnAbf62A*-m2,3 monitored by <sup>1</sup>H NMR spectroscopy. Peak area integration values  
675 are shown for the signals from 1,3-linked arabinofuranose (○), 1,2-linked arabinofuranose (●), and arabinose on  $\beta$ -  
676 furanose (▼),  $\alpha$ -furanose (Δ),  $\alpha$ -pyranose (■) and  $\beta$ -pyranose (□) forms, respectively.

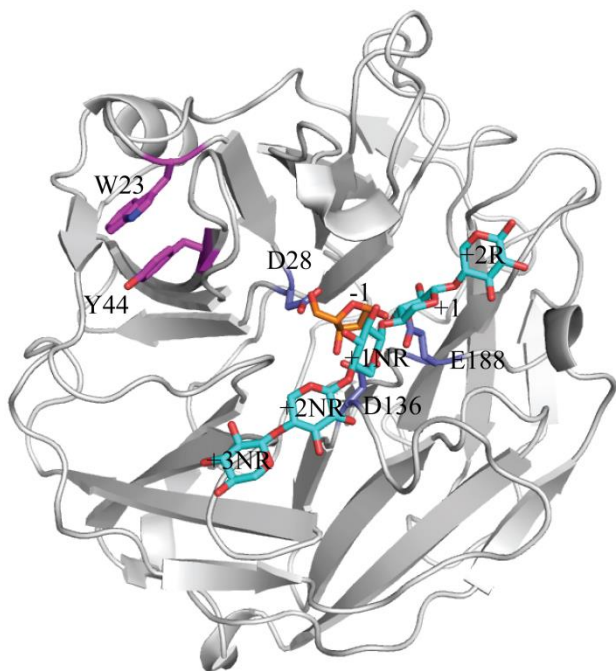
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678 **Fig. 6** Close-up surface representation of *AnAbf62A*-m2,3 putative surface binding site (SBS) situated Trp<sup>23</sup> and Tyr<sup>44</sup>  
679 (dark purple) with xylopentaose (cyan) from *ScAbf62A* (PDB ID 3WN2) and arabinose (orange) from *SthAbf62A*  
680 (PDB ID 4O8O). A) End-view from subsite +3NR on the substrate binding crevice, B) Side-view on the substrate  
681 binding crevice.

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690 **FIGURES**

691 Fig. 1



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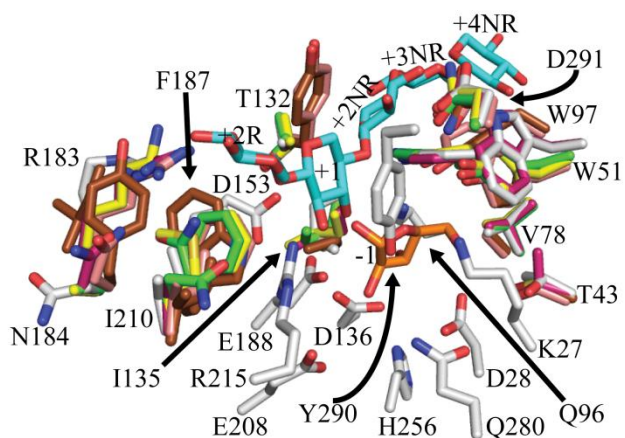
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715 Fig. 2



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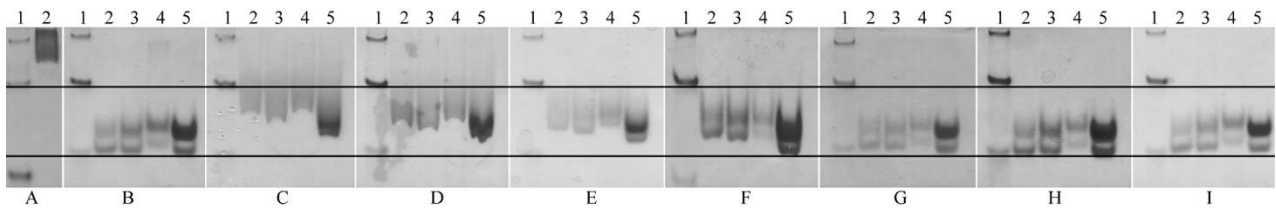
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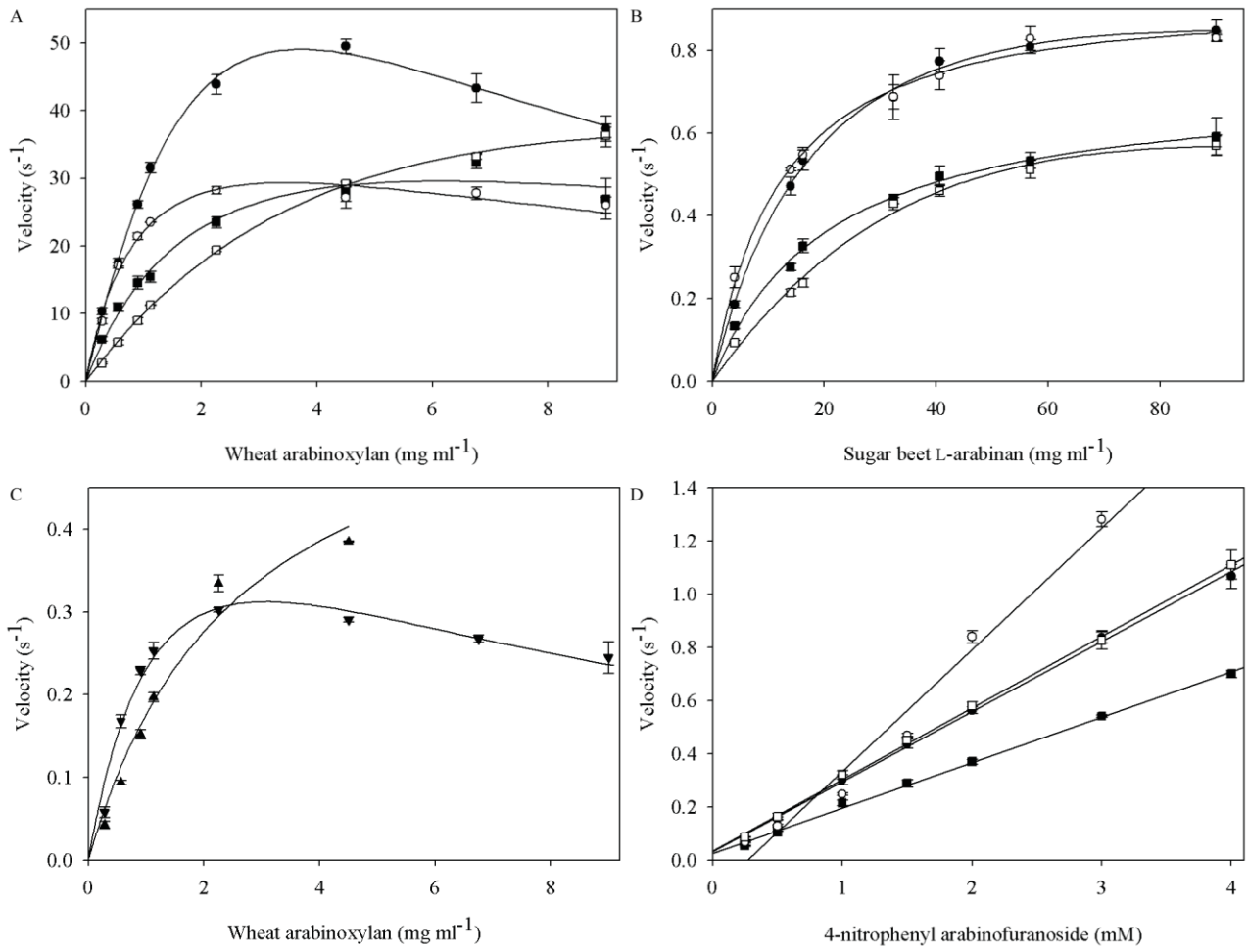
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745 Fig. 3



780 Fig. 4



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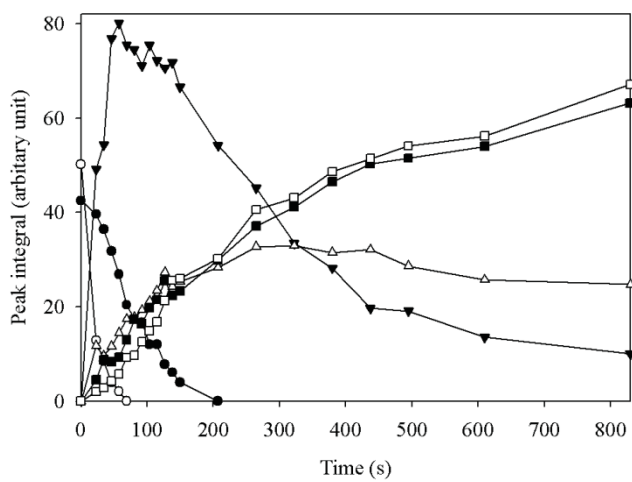
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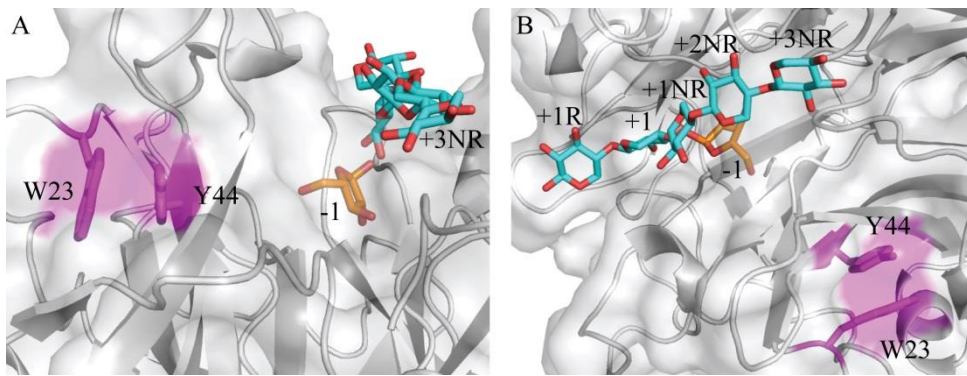
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798 Fig. 5



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827 Fig. 6



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