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Analysis of the substrate specificity of α-L-arabinofuranosidases by DNA sequencer-aided fluorophore-assisted carbohydrate electrophoresis

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Abstract

Carbohydrate-active enzymes discovery is often not accompanied by experimental validation, demonstrating the need for techniques to analyze substrate specificities of carbohydrate-active enzymes in an efficient manner. DNA sequencer-aided fluorophore-assisted carbohydrate electrophoresis (DSA-FACE) is utmost appropriate for the analysis of glycoside hydrolases that have complex substrate specificities. DSA-FACE is demonstrated here to be a highly convenient method for the precise identification of the specificity of different α-L-arabinofuranosidases for (arabino)xylo-oligosaccharides ((A)XOS). The method was validated with two α-L-arabinofuranosidases (EC 3.2.1.55) with well-known specificity, specifically a GH62 α-L-arabinofuranosidase from *Aspergillus nidulans* (*An*Abf62A-m2,3) and a GH43 α-L-arabinofuranosidase from *Bifidobacterium adolescentis* (*Ba*AXH-d3). Subsequently, application of DSA-FACE revealed the AXOS specificity of two α-L-arabinofuranosidases with previously unknown AXOS specificities. *Pa*Abf62A, a GH62 α-L-arabinofuranosidase from *Podospora anserina* strain S mat+, was shown to target the *O*-2 and the *O*-3 arabinofuranosyl monomers as side chain from mono-substituted β-D-xylosyl residues, whereas a GH43 α-L-arabinofuranosidase from a metagenomic sample only removes an arabinofuranosyl monomer from the smallest AXOS tested. DSA-FACE excels ionic chromatography in terms of detection limit for (A)XOS (picomolar sensitivity), hands-on and analysis time and the analysis of the degree of polymerization and binding site of the arabinofuranosyl substituent.

Keywords: α-L-arabinofuranosidases; substrate specificity; DSA-FACE; HPAEC-PAD; enzyme analysis.

Introduction

Carbohydrate-active enzymes (CAZymes) are often featured by a high substrate specificity that depends on the specific composition of the carbohydrate polymer, the degree and nature of substituents, and the degree of polymerization of the polymer. α-L-arabinofuranosidases (EC 3.2.1.55) (ABF) release L-arabinofuranosyl residues from arabinose-containing oligo- and polysaccharides. In particular, ABFs (also termed arabinoxylan arabinofuranohydrolases (AXHs)) active on (glucurono)arabinoxylan or their oligosaccharides can specifically target the *O*-2 and the *O*-3 arabinofuranosyl monomers from mono-substituted β-D-xylosyl residues and are therefore labeled with the suffix-m2,3. The GH62 family for example contains only ABFs-m2,3 that are active on short oligosaccharides, *para*-nitrophyl-α-L-arabinofuranoside (*p*NPA) and polysaccharides (Wilkens et al. 2017). Other ABFs are only able to cleave the *O*-3 arabinofuranosyl monomers from di-substituted β-D-xylosyl residues and are labeled with the suffix -d3, respectively (Kormelink et al. 1991a; Kormelink et al. 1991b; Pitson et al. 1996; Van Laere et al. 1999; Saha 2000; Sørensen et al. 2006; Pouvreau et al. 2011; Sakamoto et al. 2013; Wilkens et al. 2017). ABFs-d3 have only been found in the GH43 family, which is a quite diverse family in terms of substrate specificity. Mewis et al. (2016) have therefore divided the GH43 family into 37 subfamilies with subfamily 36 containing enzymes with ABF-d3 activity. ABFs that remove arabinofuranosyl monomers from both mono- and disubstituted β-D-xylosyl residues (ABF-m,d) have also been reported in GH51 (Broberg et al. 2000; Borsenberger et al. 2014) and GH54 (Sakamoto et al. 2013) families.

Analysis of (arabino)xylan-oligosaccharides ((A)XOS) produced by ABFs is generally done by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), matrix-assisted laser desorption/ionization mass spectrometry

(MALDI-TOF-MS) and nuclear magnetic resonance (NMR) (Pastell et al. 2008; Lagaert et al. 2010; Pouvreau et al. 2011; Borsenberger et al. 2014; Mccleary et al. 2015; Koutaniemi and Tenkanen 2016; Wang et al. 2017). Although these techniques are very useful for the identification of (A)XOS structures, MS- and NMR-based techniques require dedicated instrumentation, in-depth instrumental knowledge and expertise (Duus et al. 2000; Mantovani et al. 2018). HPAEC-PAD requires long analysis runs (Kabel et al. 2006) and does not always allow resolution of isomeric structures and differentiation between different patterns of substitution and molecular weights of carbohydrate oligosaccharides as shown for AXOS (Rantanen et al. 2007; Pastell et al. 2008) and arabino-oligosaccharides (Westphal et al. 2010b). Capillary electrophoresis (CE) has been proposed to be a superior method in comparison to HPAEC in terms of resolution and analysis time for the analysis of complex oligosaccharides as arabino-oligosaccharides (Westphal et al. 2010a), AXOS (Kabel et al. 2006), konjac glucomannan oligosaccharides (Albrecht et al. 2009) and xyloglucan structures (Hilz et al. 2006), allowing the study of degradation profiles of carbohydrates reacted with (putative) CAZymes by CE (Cairo et al. 2011; Alvarez et al. 2013). DNA sequencer-aided fluorophore-assisted carbohydrate electrophoresis (DSA-FACE) (later also called DNA sequencer-Assisted Saccharide analysis in High throughput, DASH), which couples the separation of fluorescently labeled oligosaccharides by CE with detection by laser-induced fluorescence, offers a valuable alternative to analyze the substrate specificity of carbohydrate-active enzymes, especially those with a complex substrate specificity (Defrancq et al. 2004; Li et al. 2013). APTS (8-aminopyrene-1,3,6-trisulfonic acid trisodium salt) is generally used as fluorescent label of the substrate or reaction products because of its negative charge, which confers electrophoretic mobility to the carbohydrates, and its compatibility with the 488-nm argon-ion laser present in many

100 standard capillary DNA sequencer devices (Evangelista et al. 1995).

101 Here, we use DSA-FACE to study the (A)XOS specificity of ABFs without the need of
102 a dedicated software and/or internal standards and compare the performance of DSA-
103 FACE to HPAEC-PAD. The method was validated by confirming the AXOS specificity
104 of a GH43 and a GH62 ABF from *Bifidobacterium adolescentis* (*Ba*AXH-d3) and
105 *Aspergillus nidulans* (*An*Abf62A-m2,3), respectively. The DSA-FACE approach is more
106 rapid and convenient than the initial methods that were used for determination of the
107 specificity (HPAEC-PAD and ^{1}H-NMR spectroscopy analysis in the case of *Ba*AXH-d3
108 (Van Laere 1997) and ^{1}H-NMR analysis and polysaccharide analysis by carbohydrate gel
109 electrophoresis (PACE) for *An*Abf62A-m2,3 (Wilkens et al. 2016). Subsequently, the
110 unknown AXOS specificities of a GH62 ABF from *Podospora anserina* (*Pa*Abf62A)
111 (39% amino acid identity with *An*Abf62A-m2,3) and a novel GH43 enzyme from a
112 metagenomic sample were identified (25% amino acid identity with *Ba*AXH-d3),
113 demonstrating the applicability of DSA-FACE to reveal precise cleavage specificity of
114 unknown ABFs in an efficient way.

115

116 **Materials and methods**

117 **Structures and abbreviations used for (A)XOS**

118 The one-letter code system proposed by Fauré et al. (2009) is used to refer to the different
119 structures of (A)XOS. The names, structures and abbreviations of the (A)XOS used in
120 this research are described in Table S1. All (A)XOS used in this research were supplied
121 by Megazyme (Megazyme International Ireland, Bray, Ireland) and have a minimum
122 purity of 95% except for $A^{2}XX$ and $A^{3}XX$, which have a minimum purity of 90%, and
123 for $XA^{2+3}XX$, which has a minimum purity of 85%.

Enzymes

The GH43 ABF from *Bifidobacterium adolescentis* (*Ba*AXH-d3, 200 U/mL, #E-AFAM2) and GH62 ABF from *Aspergillus nidulans* (*An*Abf62A-m2,3, 500 U/mL, #E-ABFAN) purified to electrophoretic homogeneity were purchased from Megazyme (Bray, Ireland). Both enzymes are produced with Megazyme recombinant strains.

*Pa*Abf62A (GenBank ID: CAP62336.1) was produced as previously described in Couturier et al. (2011).

The gene encoding a GH43 enzyme from a metagenomic sample (sequence information in note 1 of the supplementary material, GenBank ID: MH220205 from the natural sequence and MH577298 from the codon optimized sequence) without a signal peptide (aa residues 1–23) was synthesized and codon optimized for expression in *Escherichia coli* from the pET-29b+ plasmid (Genscript, NJ, USA). *E. coli* BL21 (DE3) was transformed with the corresponding plasmid for protein production. The corresponding transformant was grown at 37°C in 500 mL of Lysogeny Broth (LB) containing 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride, 50 µg/mL kanamycin, 0.5 M D-sorbitol, and 2.5 mM glycine betaine. When the cultivation reached an OD_{600} value of approximately 0.6, the culture was cooled to 15°C and induced for 16 h with 1 mM isopropyl β-D-1-thiogalactopyranoside. Cells were harvested by centrifugation at 10,000 x g for 10 min at 4°C. The pellet was then suspended in 40 mL lysis buffer (20 mM HEPES pH 7.4 and 500 mM NaCl, containing the Pierce™ protease inhibitor #A32965 (Thermo Fisher Scientific, MA, US) used according to the manufacturer's instructions) and the cells were disrupted using a EmulsiFlex-C3. Cell debris was removed by centrifugation at 12,000 x g for 20 min at 4°C.

The cell lysate was mixed with 2 mL HisTrap HP (GE Healthcare). After overnight incubation with horizontal rotation at 4°C, the matrix was transferred to a polypropylene

SPE tube with a 20 µm porosity PE frit, and connected to a Preppy™ 12-Port vacuum manifold (Sigma Aldrich Inc., SL, US). The matrix was washed with at least 10 column volumes of wash buffer (20 mM HEPES, 500 mM NaCl, pH 7.4) containing increasing concentrations of imidazole (1 mM, 5 mM, and 10 mM). Protein fractions were then collected using the wash buffer containing 25 to 500 mM imidazole. Resulting protein fractions were analyzed by 10% SDS-PAGE; selected fractions were pooled and then dialyzed against 20 mM HEPES (pH 7.4) with 10% glycerol using 30 kDa Vivaspin 20 centrifugal devices (Sartorius, Göttingen, Germany). The purified sample was flash frozen in liquid nitrogen and stored at -80°C.

Analysis of AXOS sensitivity and resolution by HPAEC-PAD

A series of dilutions between 10 µM and 0.01 µM were made for a mixture of A^2XX, $A^{2+3}XX$, XA^3XX and $XA^{2+3}XX$ in ultrapure water. Samples were filter sterilized with 0.2 µm VWR centrifugal filters with a modified nylon membrane (VWR International) and analyzed in triplicate by HPAEC-PAD using a Dionex™ ICS-3000 system (Thermo Scientific™). The ICS-3000 system is equipped with a Thermo Scientific™ Dionex™ CarboPac™ PA-100G guard column (2x50 mm), a Thermo Scientific™ Dionex™ CarboPac™ PA100 column (2x250 mm) and a pH-Ag/AgCl reference electrode. Data were analyzed with Chromeleon™ 6.8 chromatography data system software.

Mobile phase solutions were degassed through sonication for 30 min and kept at 0.250 mL/min under nitrogen during the complete run. 0.1 M NaOH was used as eluent A and 0.5 M CH_3COONa (Merck Millipore) and 0.05 M NaOH (prepared from 50% NaOH from Sigma-Aldrich) as eluent B. The elution gradient was adapted from (Rantanen et al. 2007) with the exception that the linear gradient was performed until 76% A and the second isocratic phase was done at 76% A.

174

175 **Carbohydrate labeling with APTS**

176 To analyze DSA-FACE sensitivity and resolution, an amount of 10 pmol AXOS is freeze-
177 dried and labeled with APTS (Acros Organics) as described in Callewaert et al. (2001).
178 Briefly, sugars were incubated overnight at 37°C with 1 µL of labeling solution consisting
179 of a 1:1 mixture of 20 mM APTS in 1.2 M citric acid (Acros Organics) and 1 M sodium
180 cyanoborohydride (Sigma-Aldrich) in dimethyl sulfoxide (VWR) . The labeled AXOS
181 were quenched with ultrapure water to a final concentration of 20 nM.

182

183 **Analysis of (A)XOS sensitivity and resolution by DSA-FACE**

184 A series of dilutions between 10 nM and 1.2 pM for a mixture of APTS labeled A^2XX,
185 $A^{2+3}XX$, XA^3XX and $XA^{2+3}XX$ was prepared from 10 nM AXOS labeled stocks in
186 ultrapure water. To analyze the AXOS detection limit with DSA-FACE, 10 µL of each
187 dilution was analyzed in triplicate.

188 To study the DSA-FACE capacity to resolve (A)XOS, 10 µL of 1.25 nM of a mixture of
189 APTS labeled AXOS, 1.25 nM of a mixture of APTS labeled XOS and 1.25 nM of each
190 independently APTS labeled AXOS were analyzed by DSA-FACE in triplicate.

191 DSA-FACE was performed on an Applied Biosystems™ 3130 Genetic Analyzer with 36
192 cm capillaries filled with Applied Biosystems™ POP-7™ polymer. The settings used for
193 each run are described in Table 1. The dye set chosen was the G5 dye/filter and peaks are
194 detected in the blue channel. Data were analyzed using the GeneMapper® Software
195 Version 4.0. Limits of detection (LOD) were calculated based on the linear calibration
196 curves as in Herrick (1996).

197

198 **Enzymatic reactions**

199 Ten micromolar of each non-labeled substrate was mixed with 0.2 U/mL *An*Abf62A-
200 m2,3 in 0.1 M sodium acetate buffer pH 4.5 or with 0.2 U/mL *Ba*AXH-d3 in 0.1 M
201 sodium phosphate buffer pH 6.0 or with 14.3-143 ng/mL of *Pa*Abf62A in 0.1 M sodium
202 phosphate buffer pH 6.0 or with 100 µg/mL novel GH43 enzyme from the metagenomic
203 sample in 50 mM HEPES buffer pH 7.0. Reactions were performed at 40°C and 750 rpm
204 for 3 h with *An*Abf62A-m2,3 and *Ba*AXH-d3 and for 24 h and 750 rpm with *Pa*Abf62A
205 and the novel GH43 enzyme. Buffers, pH values and reaction time were selected
206 according to the recommendations of Megazyme or empirically evaluated for completed
207 reactions.

208 All reactions were made in triplicate and were stopped by heat inactivation (80°C) for 30
209 min. A heat inactivated control, a substrate and enzyme control (in the appropriate buffer
210 for each control) were run in parallel. Enzymatic reactions with non-labeled substrate
211 were diluted to a mixture with approximately 1 µM carbohydrate, labeled and quenched
212 as described above and further diluted to 2.5-6.25 nM. Control reactions were diluted
213 similarly for comparison.

214 To obtain 100 µM of labeled AXOS for reactions with labeled substrate, ten fractions
215 containing 25 nmol sugar were freeze-dried and resuspended in 4 µL of 50 mM APTS.
216 Afterwards, labeling reactions were quenched by adding 46 µL of ultrapure water and the
217 ten aliquots were mixed. The resulting 500 µL of labeled sugar were concentrated to a
218 final volume of 100 µL by evaporation. When 2 µM labeled substrate was used, the same
219 enzyme concentration and buffer were used as above and reactions were run for 18 h.
220 Enzymatic reactions with fluorescently labeled substrate were also diluted to an
221 approximate carbohydrate concentration of 2.5 nM. 10 µL of each sample was analyzed
222 by DSA-FACE along with 1.25 nM labeled ladder of (A)XOS. There was no reaction

with 10 µM labeled substrate, which may indicate that other components present in the labeling reaction inhibit the activity of *Ba*AXH-d3 (but not AnAbf62A-m2,3). Data were analyzed and interpreted using the peak scanner (CE fragment sizing) tool of the Thermofisher Cloud.

Results

An AXOS mobility pattern can be simply inferred by DSA-FACE

A mixture of AXOS (A^2XX, A^3XX, $A^{2+3}XX$, XA^2XX, XA^3XX and $XA^{2+3}XX$) was successfully separated by DSA-FACE. The electrophoretic mobility of each sample is given in comparison to a ladder of XOS with known degree of polymerization (DP) expressed in xylose units. For (A)XOS from DP 3 to DP 6, AXOS with a DP of x have an electrophoretic mobility between x-1 and x xylose units of XOS. For instance, A^2XX, which is a xylotriose with an arabinofuranosyl substituent at the first xylosyl residue, has a DP 4 and shows an electrophoretic mobility between the xylotriose (DP 3) and xylotetraose (DP 4). This demonstrates that addition of an arabinofuranosyl substituent to a xylotriose backbone decreases the electrophoretic mobility, but less than a xylosyl residue that extends the same backbone (Fig. 1). Additionally, the *O*-3 arabinofuranosyl substituent (e.g. A^3XX, Fig. 1) decreases the electrophoretic mobility slightly more than the corresponding *O*-2 arabinofuranosyl substituent (A^2XX, Fig. 1). The effect differences between an *O*-2 and *O*-3 substituent on mobility become more pronounced for XA^2XX and XA^3XX, which have a higher DP and a substituent at the second xylosyl residue, resulting in a further improved resolution (Fig. 1). The *O*-2 and *O*-3 double arabinofuranosyl substituents have a larger effect than the mono-substituents. This effect on mobility is again significantly less pronounced than the extension of the same backbone with one xylosyl residue (e.g. $A^{2+3}XX$ and $XA^{2+3}XX$, Fig. 1).

For comparison, the same AXOS were also analyzed with HPAEC-PAD. Here, like with DSA-FACE, AXOS with a single arabinofuranosyl substituent have a lower retention time than the corresponding AXOS with double substituents, e.g. A^2XX and A^3XX elute before $A^{2+3}XX$ (Figure S1). Also like with DSA-FACE, AXOS with same DP but with an *O*-2 substituent show a lower retention time than the ones with a *O*-3 substituent (e.g. A^2XX and A^3XX) (Figure S1). However, $A^{2+3}XX$ shows a longer retention time than $XA^{2+3}XX$, although it has a lower DP (Figure S1).

DSA-FACE has a detection limit in the picomolar (pM) range and is a reproducible method for AXOS profiling

The sensitivity for AXOS detection was compared between the PAD and the fluorescence detection coupled to the capillary electrophoresis system. A dilution series of a mixture of AXOS was analyzed in triplicate with both techniques. In case of DSA-FACE, there is a linear response between 78 pM and 625 pM with a correlation coefficient of approximately 0.99 for all AXOS (Figure S2). In the non-linear region the fluorescence of $A^{2+3}XX$ is significantly higher than the fluorescence of other AXOS tested ($P<0.01$), indicating a better APTS-labeling efficiency or better excitation. The PAD response is linear for AXOS between 0.3 and 10 μM and equal for all compounds with a high correlation coefficient for all AXOS (>0.99) (Figure S3). For the AXOS studied in ultrapure water, the limit of detection (LOD) varied for DSA-FACE from 38 pM ($XA^{2+3}XX$) to 55 pM (A^2XX), whereas for HPAEC-PAD from 51 nM (XA^3XX) to 126 nM (A^2XX) (Table 2). It can thus be concluded that DSA-FACE is at least 10^3 times more sensitive than HPAEC-PAD.

The repeatability in terms of electrophoretic mobilities/retention times of the DSA-FACE and HPAEC-PAD, respectively, was compared for different concentrations of AXOS in

ultrapure water. In general, the coefficients of variation for both techniques are low and both DSA-FACE and HPAEC-PAD show a similar repeatability (Tables S2 and S3).

DSA-FACE requires less hands-on time and analysis time than HPAEC-PAD

In terms of hands-on and analysis time, DSA-FACE outperforms HPAEC-PAD to analyze AXOS profiles. When using HPAEC-PAD it is necessary to regenerate and equilibrate the resin at the start of each run, which takes a considerable amount of time. The DSA-FACE on its turn does not need any regeneration/equilibration step and does not require a regular maintenance as is the case for HPAEC-PAD since the CE polymer is replaced between each analysis reducing the risk of cross-contamination. Samples for HPAEC-PAD require filtering, whereas samples for DSA-FACE must be labeled. In total, DSA-FACE has an about 3x shorter hands-on time and a 3-7x faster analysis per four samples compared to HPAEC-PAD (Table 2).

DSA-FACE is a convenient method to reveal α-L-arabinofuranosidases substrate specificity

The substrate specificity was first analyzed by DSA-FACE for two commercially available, recombinant ABFs (*Ba*AXH-d3 and *An*Abf62A-m2,3) with known specificities. Both enzymes were incubated with substrates with a single or double substituent on the non-reducing xylosyl residue (A^2XX, A^3XX and $A^{2+3}XX$) or at the second xylosyl starting from the non-reducing end (XA^2XX, XA^3XX and $XA^{2+3}XX$). The reaction mixtures were analyzed by DSA-FACE and compared with the electrophoretic mobility of an (A)XOS ladder. *Ba*AXH-d3 is only active on double substituted xylosyl residues as $A^{2+3}XX$ and $XA^{2+3}XX$ (Fig. 2a) and generates A^2XX and XA^2XX after reaction, respectively. It should be noted that the peak corresponding to the released

298 arabinose has a too high electrophoretic mobility to be observed. *An*Abf62A-m2,3
299 completely converts A^2XX to xylotriose and XA^2XX and XA^3XX to xylotetraose (Fig.
300 2b), respectively. Similar to *Ba*AXH-d3, *An*Abf62A-m2,3 is not affected by the non-
301 reducing end xylosyl (Fig. 2b). DSA-FACE could thus successfully validate these
302 substrate specificities, but with a less laborious approach than for their initial
303 identification.
304 Subsequently, two ABFs with unknown AXOS substrate specificities were selected.
305 DSA-FACE analysis of *Pa*Abf62A with different specific AXOS demonstrated that
306 *Pa*Abf62A can hydrolyze *O*-2 and *O*-3 arabinofuranosyl substituents from A^3X, A^2XX,
307 A^3XX, XA^2XX, XA^3XX (Fig. 3). *Pa*Abf62A does not have a preference for a non-
308 reducing end arabinofuranosyl residue or for one at an internal xylosyl residue. Notably,
309 it was not possible to completely inactivate this enzyme at 80°C for 30 min as seen in the
310 heat inactivated controls, indicating a high thermostability (Fig. 3, in the case of A^2XX,
311 A^3XX, XA^2XX and XA^3XX).
312 A second ABF with unknown substrate specificity was selected from a metagenomic
313 sample isolated from pulp mill anaerobic granules enriched for over four years on
314 pretreated poplar wood fiber (unpublished results). The GH43 ABF was identified
315 following CAZyme assignments of the assembled metagenome, as reported in Wong et
316 al. (2017). A^3X, a mixture of A^2XX and A^3XX and XA^2XX and XA^3XX, $A^{2+3}XX$ and
317 $XA^{2+3}XX$ were used as substrate for the novel GH43 ABF and the reaction mixture was
318 analyzed with DSA-FACE. The only accepted substrate was the smallest substrate (A^3X),
319 which was partially converted to xylobiose (Fig. 4 and Figure S4).
320 *Ba*AXH-d3 (Figure S5), *An*Abf62A-m2,3 (Figure S6), *Pa*Abf62A (Figure S7) and the
321 novel GH43 ABF identified from a metagenomic sample (Figure S8) were also analyzed
322 after reaction with XOS (XXXX, XXXXX and XXXXXX and also XX and XXX for the

323 novel GH43 enzyme), and they all showed no endo-xylanase activity since the XOS
324 hydrolysates electropherograms remain unchanged compared to the substrate and heat
325 inactivated controls.
326
327 **AXH-d3 α-L-arabinofuranosidases hydrolysates must be labeled after hydrolysis**
328 In terms of experimental set-up and enzyme kinetics it would be advantageous if the
329 enzymatic reaction could also be performed with APTS-labeled substrate. A prior
330 labeling of AXOS would significantly reduce the hands-on time after the enzymatic
331 reactions as only a limited number of AXOS stocks must be labeled. In this particular
332 case, it would reduce the overall hands-on time for DSA-FACE analysis to approximately
333 0.5 h in case of the analysis of four samples (Table 2). The background in the
334 electropherograms would also be reduced since only a pure substrate would be labeled
335 and not the whole hydrolysate including enzyme and buffer components.
336 *Ba*AXH-d3 activity on $A^{2+3}XX$ is clearly affected by the label at the reducing end of the
337 sugar. The enzyme hydrolyzes the *O*-3 arabinofuranosyl substituent but another peak with
338 DP 4 is also present (Fig. 5). Different trials by spiking with XXXX, $A^{2+3}XX$, $A^{2}XX$ and
339 $A^{3}XX$ did not give a reliable identification of the additional peak (data not shown). In
340 contrast, the *An*Abf62A-m2,3 substrate specificity on $A^{2}XX$ is not affected by the APTS
341 labels since the same electrophoretic mobility profiles are obtained for both enzymatic
342 reactions (Figure S9). The APTS has thus only an influence on the AXH-d3 reaction
343 which might indicate that APTS changes the interaction between the *O*-2 and/or *O*-3
344 arabinofuranosyl substituents of the substrate and the active site of the enzyme and/or the
345 orientation of the substrate towards the enzyme.
346
347

Discussion

We have presented here DSA-FACE as a convenient method to analyze the AXOS specificity of ABFs. Our approach is based on the AXOS mobility pattern that can be easily inferred by DSA-FACE. The electrophoretic mobility of AXOS generally decreases with their DP, but the nature of the substituent affects this decrease (Fig. 1). The substituent effects can be explained by differences in hydrodynamic volume, even when the charge to mass ratio of these carbohydrates is the same. Hydrodynamic volume of sugars differs depending on DP and type of linkages (Herrick 1996; Mittermayr and Guttman 2012), but it cannot be excluded that also internal interactions, depending on the position of the substituents, may influence the charge to mass ratio and thus the mobility. When analyzing AXOS by HPAEC-PAD, no set of easy rules could be defined to reveal the AXOS structure in contrast to DSA-FACE. Therefore, DSA-FACE is more appropriate to study AXOS substrate specificity of ABFs than HPAEC-PAD.

DSA-FACE can detect as low as 38 pM (picomolar range) of released AXOS after labeling, which allows the study of substrate specificities of enzymes available in small amounts or to detect minor activities. DSA-FACE is approximately 10^3 more sensitive than HPAEC-PAD (nanomolar range). The repeatability of DSA-FACE data is high, however, there is some remaining variability that is likely explained by the electrokinetic injection mechanism of the samples. Factors like temperature, sample matrix, viscosity of the polymer and presence of protein in the matrix affect electrokinetic injection and consequently migration times and peak areas vary from run to run (Sepaniak 2000).

*Ba*AXH-d3 and *An*Abf62A-m2,3 with known substrate specificities were used as a proof of concept to show the applicability of DSA-FACE in the study of the substrate specificities of ABFs. The substrate specificity of native *Ba*AXH-d3, a GH43 α-L-arabinofuranosidase from *Bifidobacterium adolescentis* was earlier described with the

help of HPAEC-PAD and ^{1}H-NMR (Van Laere et al. 1997; Van Laere et al. 1999). Native *Ba*AXH-d3 releases *O*-3 arabinofuranosyl residues from *O*-2 and *O*-3 doubly-substituted xylosyl monomers from wheat flour arabinoxylan, $A^{2+3}XX$ and $XA^{2+3}XXX$ but not from single-substituted AXOS, soy arabinogalactan and sugar-beet arabinan and their oligosaccharides. While native *Ba*AXH-d3 apparently shows no detectable activity towards *p*NPA, recombinant *Ba*AXH-d3 was able to release *p*-nitrophenol from this substrate at a very low rate (van den Broek et al. 2005). *An*Abf62A-m2,3, a recombinant GH62 α-L-arabinofuranosidase from *Aspergillus nidulans*, removes both *O*-2 and *O*-3 arabinofuranosyl substituents from single-substituted xylosyl monomers of AXOS and AX as determined by ^{1}H-NMR analysis and polysaccharide analysis by carbohydrate gel electrophoresis (PACE) (Wilkens et al. 2016). From the (A)XOS studied, *Ba*AXH-d3 is only active on double substituted xylosyl residues as $A^{2+3}XX$ and $XA^{2+3}XX$. *O*-3 linked arabinofuranosyl substituents are removed and the non-reducing end xylosyl present in $XA^{2+3}XX$ does not inhibit efficient arabinose removal. *An*Abf62A-m2,3 was proved to remove the *O*-2 and *O*-3 linked arabinofuranosyl substituents and not to be affected by the non-reducing end xylosyl, as well (Fig. 2b). DSA-FACE could thus successfully validate these substrate specificities, but with a less laborious approach than for their initial identification. Subsequently, the substrate specificity of *Pa*Abf62A was for the first time demonstrated with (A)XOS by DSA-FACE. *Pa*Abf62A was identified before as a GH62 ABF in the genome of the ascomycete *Podospora anserina*, a coprophilous fungus acting on recalcitrant polysaccharides (Couturier et al. 2016).

Its crystal structure was determined in complex with arabinose and cellotriose (PDB 4N2Z, 4N4B) (Siguier et al. 2014). Weak arabinofuranosidase activity was detected with the chromogenic substrate pNPA. In addition, it was shown with HPAEC-PAD that PaAbf62A releases solely arabinose from wheat arabinoxylan and sugar beet arabinan

and not from debranched or linear arabinan (Wong et al. 2017). PaAbf62A could now be specified as ABF-m2,3, removing O-2 and O-3 arabinofuranosyl substituents of monosubstituted AXOS. Similar to PaAbf62A, the GH43 ABF was shown before to release arabinose from pNPA, however, substrate preferences using AXOS were still unknown. An unusual substrate specificity for a small substrate (A^3X) was discovered for this novel enzyme identified from a metagenomic sample using DSA-FACE. Sequence alignments (Blastp) between the metagenomic GH43 enzyme and the 154 characterized GH43 enzymes in the CAZymes database revealed only four significant hits with coverages between 93% and 85%: two arabinofuranosidases from *Bacteroides thetaiotaomicron* VPI-5482 (accession numbers AAO78760.1 and AAO76128.1) to which the metagenomic GH43 sample shares 27% and 45% identity, respectively; an endo-1,4-β-xylanase from *Bifidobacterium adolescentis* ATCC8483 (accession number BAF40308.1) with 25% shared identity and a glycosyl hydrolase from *Bacteroides ovatus* ATCC 8483 (accession number EDO10792.1) with a shared identity of 26%. The arabinofuranosidases from *Bacteroides thetaiotaomicron* VPI-5482 belong to subfamilies 19 and 18 and the endo-1,4-β-xylanase and the *Bacteroides ovatus* glycosyl hydrolase belong to subfamilies 22 and 12, respectively. Due to their low homology to the metagenomic GH43 enzyme and their variability in substrate specificity and subfamily classification, an accurate prediction on the subfamily classification and substrate specificity of the metagenomic GH43 enzyme is not possible.In earlier reports, specificities have sometimes been determined with labeled substrates (Wang et al. 2011; Eda et al. 2014). Although the use of labeled substrates would save a significant amount of time and reduce the background signal, caution should be taken since prior labeling of the substrates may bias the reaction outcome, resulting in a misannotation of the enzyme specificity.

The Applied Biosystems™ 3130 Genetic Analyzer used for the DSA-FACE analyses offers the possibility to work in high-throughput. The presented method can be operated in a 96-well plate format in around 14 h with the settings applied to analyze (A)XOS. Overall, DSA-FACE can reveal the substrate specificity of ABFs without the use of an internal standard, with a shorter analysis and hands-on time in comparison to HPAEC-PAD and using representative AXOS. The convenience and the throughput potential of DSA-FACE can accelerate the study of enzymatic activities by analyzing, for example, a high number of putative enzymes from metagenomic samples or after directed evolution experiments. In addition, it can also be of help to study the influence of different substrate structures or different reaction conditions for a single enzyme.

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Compliance with ethical standards

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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632 **Figure captions**

633 **Fig. 1** DSA-FACE electropherograms of (A)XOS. Electrophoretic mobility pattern of a
634 mixture of XOS with DP 3 to 6 was compared to A^2XX, a mixture of A^2XX and A^3XX,
635 $A^{2+3}XX$, XA^3XX, a mixture of XA^2XX and XA^3XX and $XA^{2+3}XX$. In yellow, green and
636 pink are represented the DP regions of AXOS

637

638 **Fig. 2** DSA-FACE analysis of *Ba*AXH-d3 (a) and *An*Abf62A-m2,3 (b) hydrolysates.
639 Electropherograms of reactions with *Ba*AXH-d3 and *An*Abf62A-m2,3 and A^2XX,
640 $A^{2+3}XX$, XA^3XX, a mixture of XA^2XX and XA^3XX and $XA^{2+3}XX$. Control reactions
641 with heat inactivated enzyme, substrate and enzyme alone were included. All reactions
642 per enzyme were performed under the same reaction conditions

643

644 **Fig. 3** DSA-FACE analysis of *Pa*Abf62A hydrolysates. Electropherograms of reactions
645 with *Pa*Abf62A and A^3X, a mixture of A^2XX and A^3XX, a mixture of XA^2XX and
646 XA^3XX, $A^{2+3}XX$ and $XA^{2+3}XX$. Control reactions with enzyme incubated at 80°C,
647 substrate and enzyme alone were included. All reactions were performed under the same
648 reaction conditions

649

650 **Fig. 4** DSA-FACE analysis of hydrolysates of a novel GH43 enzyme identified from a
651 metagenomic sample. Electropherograms of the hydrolysates obtained after incubation of
652 GH43 enzyme generated from metagenomic data and A^3X and a mixture of A^2XX and
653 A^3XX. Control reactions with heat inactivated enzyme, substrate and enzyme alone were
654 included. All reactions were performed under the same reaction conditions

655

Fig. 5 DSA-FACE analysis of *Ba*AXH-d3 hydrolysates when incubated with APTS-labeled $A^{2+3}XX$. Electropherograms of *Ba*AXH-d3 and APTS-labeled and non-labeled $A^{2+3}XX$. Control reactions with enzyme incubated at 80°C, substrate and enzyme alone were included. Question mark is the unknown peak that appears after reaction with *Ba*AXH-d3 and APTS-labeled $A^{2+3}XX$

Tables

Table 1 Applied Biosystems™ 3130 Genetic Analyzer settings. All (A)XOS were run under the following conditions

Parameter	Value
Oven temperature	60°C
Current stability	5 µA
Pre-run voltage	15 kV
Pre-run time	180 s
Injection voltage	1.2 kV
Injection time	16 s
Voltage nº of steps	20 nk
Voltage step interval	15 s
Data delay time	60 s
Run voltage	15 kV
Run time	1200 s

671 **Table 2** HPAEC-PAD and DSA-FACE comparison in terms of resolution, sensitivity,
672 repeatability and total hands-on-time and analysis time. Hands-on time and analysis time
673 are calculated for the analysis of 4 samples
674

Technique / Parameter	HPAEC-PAD	DSA-FACE
Resolution	Customized elution programs must be chosen for the complete separation of (A)XOS	Good separation for all (A)XOS studied
LOD	From 51 nM to 126 nM for the AXOS studied	From 38 pM to 55 pM for the AXOS studied
Retention time/ electrophoretic migration repeatability	Coefficient of variation: 0.09% - 3.45%,	Coefficient of variation: 0.3%
Hands-on time	- Prepare and degas elution eluents **(~1.3 h)** - Regenerate and equilibrate column **(~3 h)** - Samples (dilution and) filter sterilization **(~0.7 h)** - Start analysis **(~0.4 h)** **Total time:** ~5.4 h	- Prepare labeling solution **(~0.5 h)** - Dilute samples to ~1 µM **(~0.25 h)** - Labeling reaction **(~0.3 h)** - Stop labeling reaction and start the run **(~0.7 h)** **Total time:** ~1.75 h
Analysis time	**~2 to 4 h** (elution program dependent)	**~0.6 h**

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