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Mode of action of *Chrysosporium lucknowense* C1 α -L-arabinohydrolases

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ABSTRACT

The mode of action of four *Chrysosporium lucknowense* C1 α -L-arabinohydrolases was determined to enable controlled and effective degradation of arabinan. The active site of endoarabinanase Abn1 has at least six subsites, of which the subsites –1 to +2 have to be occupied for hydrolysis. Abn1 was able to hydrolyze a branched arabinohexaose with a double substituted arabinose at subsite –2. The exo acting enzymes Abn2, Abn4 and Abf3 release arabinobiose (Abn2) and arabinose (Abn4 and Abf3) from the non-reducing end of reduced arabinose oligomers. Abn2 binds the two arabinose units only at the subsites –1 and –2. Abf3 prefers small oligomers over large oligomers. It is able to hydrolyze all linkages present in beet arabinan, including the linkages of double substituted residues. Abn4 is more active towards polymeric substrate and releases arabinose monomers from single substituted arabinose residues. Depending on the combination of the enzymes, the C1 arabinohydrolases can be used to effectively release branched arabinose oligomers and/or arabinose monomers.

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1. Introduction

Sugar beet pulp consists of up to 75% (w/w) carbohydrates on dry matter basis (McCready, 1966). Up to one third of the carbohydrate material is polymeric arabinan, a heavily branched, complex neutral sugar side chain of pectic rhamnogalacturonan I (Rombouts et al., 1988). Sugar beet arabinan has a backbone of linear α -1,5-linked L-arabinofuranose units. Monomeric or oligomeric L-arabinofuranose branches are linked to the backbone via α -1,2-linkages and α -1,3-linkages. The oligomer side chains may even be further branched. A number of researchers report that up to 1% of the arabinose is present as terminal L-arabinopyranose (summarized in Beldman et al. (1997)).

Arabinans play a great role in food industry. Polymeric arabinans isolated from beet pulp are able to form spreadable gels and act as fat replacers. They are considered dietary fibers due to their resistance to human digestive enzymes (Cooper et al., 1992; McCleary et al., 1995). Furthermore, arabinose oligomers have been reported to have prebiotic effects on intestinal bacteria in the distal colon (Van Laere et al., 2000, 2002). More complex oligomers could have advanced prebiotic properties due to lower digestibility in the proximal colon. L-Arabinose is considered as a functional ingredient because it inhibits intestinal sucrose, leading to decreased plasma glucose levels (Seri et al., 1996; Osaki et al., 2001).

The degradation of the heavily branched sugar beet arabinan requires the concerted action of a number of powerful arabinohydrolases. Arabinohydrolases are a group of enzymes that belong to the glycoside hydrolase families GH 3, 27, 43, 51, 54, 62 and 93 (<http://www.cazy.org>), of which GH family 3, 43, 51, 54, 62 and 93 encode for α -L-arabinohydrolases. One β -L-arabinopyranohydrolase described so far is grouped into GH family 27 (Ichinose et al., 2009).

Endoarabinanases (EC 3.2.1.99) belong to GH family 43. They hydrolyze the α -1,5-linkages in the unsubstituted regions of the arabinan backbone with an inverting mode of action (Alhassid et al., 2009). Endoarabinanases prefer polymers over oligomers. Their activity decreases with decreasing degrees of polymerization (Dunkel and Amado, 1995). Arabinotriose is the smallest substrate for the enzyme and it is hydrolyzed to arabinose and arabinobiose upon end point hydrolysis (Rombouts et al., 1988). Structural studies of a GH family 43 endoarabinanase from *Geobacillus stearothermophilus* revealed that the substrate binding site is a binding cleft that can harbor at least five arabinose units in which arabinotriose occupies the subsites –1 to +2 (Alhassid et al., 2009).

Exoarabinanases belong to GH families 43 and 93 (EC 3.2.1.-) and release arabinose, arabinobiose or arabinotriose from the non-reducing end of the α -1,5-linked arabinan backbone (Kaji and Shimokawa, 1984; Sakamoto and Thibault, 2001; Ichinose et al., 2008). All of these enzymes specifically release only one product and preferably act on linear arabinan. The conformation of the substrate binding site greatly determines the enzymes mode of action. Proctor et al. (2005) could change the mode of action of GH family 43 exoarabinanase 43A from *Cellvibrio japonicus* into an endo-mode of action by redesigning its binding site.

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Arabinofuranosidases (EC 3.2.1.55) active towards arabinan belong to the GH families 3, 43, 51, 54 and 62 (<http://www.cazy.org>). They are able to release arabinose monomers from the non-reducing ends of arabinan. GH family 3 α -L-arabinofuranosidases are bifunctional enzymes that also act as β -D-xylosidases (<http://www.cazy.org>). GH family 62 arabinofuranosidases are reported to be predominantly active towards arabinoxylan and are, therefore, also called arabinoxylan arabinofuranohydrolases (Beldman et al., 1997). Besides their structural classification, arabinofuranosidases are classified according to their substrate specificity into arabinofuranosidases A + B and arabinofuranohydrolases specific towards arabinoxylans (Beldman et al., 1997). Arabinofuranosidase A (AbfA) and arabinofuranosidase B (AbfB) are both active towards arabinose oligomers and *p*-nitrophenyl arabinofuranoside (*p*NP-Araf). Their activities differ towards polymeric substrates as AbfA is inactive towards linear arabinan and much less active than AbfB towards branched arabinan (Rombouts et al., 1988).

The genome of the ascomycete *Chrysosporium lucknowense* (C1) encodes 14 enzymes that putatively release arabinose or arabinose oligomers from arabinan (Hinz et al., 2009). We have recently purified and characterized four α -L-arabinohydrolases that specifically and synergistically act on the degradation of sugar beet arabinan (Kühnel et al., 2010). A mixture of linear and branched arabinose oligomers was released when branched arabinan was digested with endoarabinanase Abn1, exoarabinanase Abn2 and arabinofuranosidase Abn4 (Westphal et al., 2010a). No detailed information is available about the mechanism how the C1 arabinohydrolases act in synergy. This information is necessary to ensure a controlled and efficient degradation of the substrate.

Well characterized enzymes are the prerequisite for the directed release of arabinose oligomers. Therefore, in this research branched arabinose oligomers and reduced linear arabinose oligomers were used to determine the substrate specificity and mode of action of the C1 endoarabinanase Abn1, exoarabinanase Abn2 and the two arabinofuranosidases Abn4 and Abf3.

2. Methods

Enzyme substrate binding subsites were named following the nomenclature for sugar-binding subsites in glycosyl hydrolases (Davies et al., 1997). Centrifugation was always performed at room temperature at 15,000g for 10 min.

2.1. Material

The C1 enzymes Abn1, Abn2, Abn4 and Abf3 were purified previously by Kühnel et al. (2010). Their activities are given in Table 1. Linear arabinose oligomers in the range from DP 2–7 used for incubations and for the production of reduced arabinose oligomers were purchased from Megazyme (Megazyme; Bray, Ireland). Linear and branched sugar beet arabinan were from British Sugar (Peterborough, United Kingdom). Pullulan molecular mass standards were from Polymer Laboratories (Varian Inc., Palo Alto, CA, USA). Branched arabinose oligomers were produced, purified and charac-

Table 1
Activities of C1 α -L-arabinohydrolases as determined previously (Kühnel et al., 2010). Linear and branched arabinan from sugar beet. 1 U = 1 μ mol (product)/min mg (enzyme).

	GH family	Activity (U/mg)	Substrate
Abn1	GH 43	26.0	Linear arabinan
Abn2	GH 93	7.1	Linear arabinan
Abn4	GH 43	9.5	Branched arabinan
Abf3	GH 51	21.4	<i>p</i> NP-Araf

terized by Westphal et al. (2010a). Other chemicals were from Merck or Sigma–Aldrich.

2.2. Action of C1 arabinohydrolases towards reduced arabinose oligomers

2.2.1. Production of reduced arabinose oligomers

Arabinose and arabinose oligomers in the range from DP 2–7 (5 mg each) were reduced with 7.5 mg sodium borohydride in 0.2 ml ammonium hydroxide (1.5 M) for 1 h at ambient temperature. The reaction was stopped by the addition of glacial acetic acid until gas formation ceased (pH < 5.0). Methanol (1 ml) was added to remove the sodium borohydride as volatile trimethylborate and the samples were dried under a stream of air. The methanol washing was repeated two times. Samples were dissolved in water and the reduction was verified by HPAEC (Section 2.5).

2.2.2. Action of C1 arabinohydrolases towards reduced arabinose oligomers

Pure, reduced arabinose oligomers in the range from DP 2–7 (2 mg/ml, 25 μ l) were mixed with 25 μ l enzyme solution in 100 mM sodium acetate buffer (Abn1 and Abn2: 0.35 mU, Abn4: 0.3 mU and Abf3: 0.4 mU). The samples were incubated for 1, 15 and 20 h ($T = 30^\circ\text{C}$) with an additional dose of fresh enzyme (4 mU in 10 μ l) added after 15 h. After incubation, the samples were diluted 20 times, boiled ($t = 5$ min) and centrifuged. The supernatant was analyzed by HPAEC (Section 2.5).

2.3. C1 arabinohydrolase activities towards branched arabinose oligomers

Branched arabinose oligomers (0.1 mg) were incubated for 2 h at 30°C with Abn1 (30 mU), Abn4 (20 mU) or Abf3 (20 mU) in a total volume of 100 μ l. After incubation, the samples were diluted with 400 μ l water and boiled for 5 min. After centrifugation, the supernatant was analyzed by PGC–HPLC–MS (Section 2.5).

2.4. Product inhibition of C1 arabinohydrolases

2.4.1. Abn1 and Abn2

Product inhibition of Abn1 and Abn2 was studied towards linear arabinan in the presence of arabinobiose as the end product for both enzymes. Abn1 and Abn2 (6 mU) were incubated for 1 h at 30°C with 0.5 mg linear arabinan and arabinobiose (0, 0.1, 0.5, 1, 2, 3, 4 and 5 mg) in 100 μ l total volume. The samples were subsequently boiled ($t = 5$ min) and centrifuged. The supernatant was transferred into a HPLC vial and subjected to HPSEC analysis (Section 2.5).

2.4.2. Abn4 and Abf3

The product inhibition of Abn4 and Abf3 was studied towards *p*NP-Araf in the presence of arabinose. Abn4 (0.5 mU) and Abf3 (1 mU) were incubated for 30 min at 30°C in a microtiter plate in 200 μ l total volume, including 10 mM sodium acetate buffer (pH 5.0), 0.5 mM *p*NP-Ara and arabinose monomer concentrations from 0 to 500 mM. The pH was adjusted to pH 10.0 with 50 μ l sodium carbonate (0.5 M) and the amount of free *p*-nitrophenol was determined photospectrometrically at 405 nm. A *p*-nitrophenol standard curve (10–500 μ M in 100 mM sodium carbonate) was used for quantification. Abf3 inhibition kinetics were studied with a slightly modified protocol: $t = 15$ min, $V = 200$ μ l: 100 μ l *p*NP-Ara (0.25–2.5 mM), 50 μ l arabinose (2 M) or water, 40 μ l sodium acetate buffer (50 mM, pH 5.0) and 10 μ l Abf3 (1 mU).

2.5. Chromatography

High performance size exclusion chromatography (HPSEC) was performed on a Dionex Ultimate 3000 System (Dionex, Sunnyvale, CA, USA) equipped with a set of four TSK-Gel superAW columns (Tosoh Bioscience, Tokyo, Japan) in series: guard column (6 mm ID \times 40 mm) and separation columns 4000, 3000 and 2500 (6 mm ID \times 150 mm). The samples (10 μ l; 5 mg/ml) were eluted with filtered aqueous 0.2 M sodium nitrate at 40 °C at a flow rate of 0.6 ml/min. Elution was followed by refractive index detection (Shodex RI 101; Showa Denko K.K., Kawasaki, Japan).

The monomer and oligomer carbohydrate levels of the digests were analyzed by high performance anion exchange chromatography (HPAEC) according to Albrecht et al. (2009) using a CarboPac PA1 analytical column (2 \times 250 mm, Dionex) in combination with a CarboPac PA1 guard column (2 \times 50 mm, Dionex). Arabinose and arabinose oligomers (10 μ l; 50–100 μ g/ml) were eluted in 0.1 M sodium hydroxide with an adapted sodium acetate (NaOAc) elution profile: a gradient of 0–350 mM NaOAc over 30 min, 1 M NaOAc for 10 min and 0 M NaOAc for 15 min (equilibration).

Branched oligomers were separated and identified by PGC-HPLC-ELSD/MS according to Westphal et al. (in press). A Thermo Accela UHPLC System (Waltham, MA, USA) equipped with a Hypercarb column (PGC, 100 \times 2.1 mm; 3 μ m, Thermo Electron Corporation, San José, CA, USA) was used in combination with a Hypercarb guard column (10 \times 2.1 mm; 3 μ m, Thermo Electron Corporation). Samples (10 μ l; c = 100–400 μ g/ml) were eluted at 0.4 ml/min and 70 °C with an elution profile consisting of three eluents: 25 μ M lithium acetate (LiAc) in Millipore water (A), 25 μ M LiAc in acetonitrile (B) and 25 μ M LiAc in 0.2% (w/v) aqueous trifluoroacetic acid (C). Elution profile: 0–1 min: 100% A, 1–15 min: linear gradient from 0% to 27.5% B, 15–28 min: linear gradient from 27.5% to 60% B and from 0% to 10% C, 28–31 min: linear gradient from 60% to 80% B and from 10% to 20% C, 31–35 min: 80% B and 20% C, 35–36 min: gradient from 80% B and 20% C to 100% A, 36–41 min: equilibration with 100% A. The PGC-column was coupled to a 1:1-splitter (Accurate, Dionex) directing the eluent both to an ELSD85 evaporative light scattering detector (Sedere, Alfortville, France) and to an ESI-MSⁿ-detector (LTQ XL MS, ion trap, Thermo Electron Corporation). The drift tube temperature of the ELSD was set to 50 °C and the gain to 12. MS-detection was performed in positive mode (spray voltage: 4.6 kV, capillary temperature: 260 °C, auto-tuned on arabinopentaose (m/z = 685)). The ion trap was closed either after 10 ms or when intensity of 3×10^5 was reached to enable the detection of minor components and to avoid overload of the MS by abundant components.

3. Results and discussion

3.1. Mode of action of C1 arabinohydrolases towards reduced arabinose oligomers

3.1.1. Abn1

The action of α -L-endoarabinanase Abn1 towards reduced arabinose oligomers is shown in Fig. 1. Abn1 degraded reduced arabinose oligomers with DP \geq 3 and showed increasing activities with increasing degrees of polymerization (Fig. 1A). Reduced arabinobiose was the smallest labeled compound identified in all digests and it was the first product formed in DP 3–5 oligomer digests (no further data shown). All produced oligomers with DP \geq 3 were subject to further hydrolysis that yielded arabinose, arabinobiose and reduced arabinobiose as final products. The hydrolytic performance of Abn1 did not linearly increase with increasing DP. It increased stepwise from DP 4 to 5 and from DP 6 to 7, respectively, and remained similar from DP 3 to 4 and from

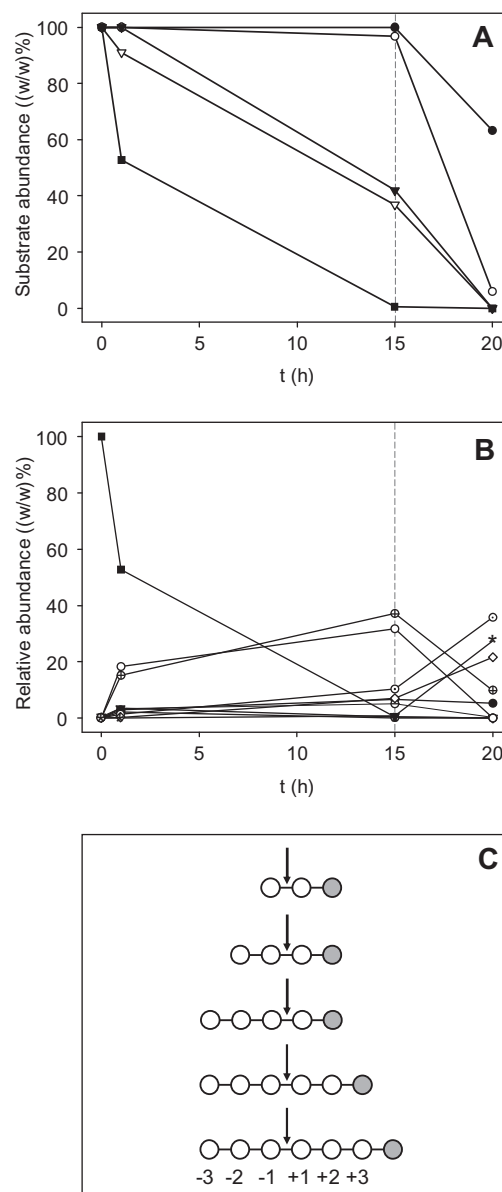


Fig. 1. Abn1 action towards reduced arabinose oligomers. (A) Time dependent consumption of pure reduced arabinose oligomers in the range from DP 3 to 7. (B) Product formation from reduced arabinose oligomers: ◇ – reduced arabinobiose, ● – reduced arabinotriose, ◊ – reduced arabinotetraose, ◻ – reduced arabinopentaose, ◼ – reduced arabinohexaose, ◼ – reduced arabinohexaose, * – arabinose, ○ – arabinobiose, ⊕ – arabinotriose, ⊖ – arabinotetraose, ⊗ – arabinopentaose, and grey dashed line – fresh 10-fold enzyme dose added after 15 h incubation. (C) Schematic mode of action of Abn1 towards reduced arabinose oligomers and main products formed after first hydrolysis step. White circles – arabinose units, grey circles – reduced arabinose, arrows – primarily hydrolyzed linkage by Abn1, numbers from –3 to +3 – predicted binding subsites of Abn1.

DP 5 to 6, respectively (Fig. 1A). The activity increase from DP 4 to DP 5 was connected to the interaction of an additional arabinose with the subsite –3 (Fig. 1C). Accordingly, the activity increase from DP 6 to 7 was linked to the interaction of an internal arabinose unit with the subsite +3. In contrast, the occupation of the subsite –2 in the DP 4 oligomer and the +2 subsite in the DP 6 oligomer, respectively, did not alter Abn1 activity. These data imply that the subsite –3 has a higher binding affinity than the subsites –2 and +3.

The reduction of the free aldehyde groups causes structural and conformational changes within the molecule. Nevertheless, Abn1

could degrade reduced arabinose heptaose with the same degree of hydrolysis and specificity as non-reduced arabinose heptaose and was only slightly less active towards reduced arabinotriose when compared to non-reduced arabinotriose (provided as [Supplementary data](#), Fig. S2). This data suggests that the action of Abn1 is not greatly influenced by the modification of the reducing end group. The data presented in Fig. 1 might further imply that the substrate binding site of Abn1 recognizes at least six arabinose units. The degradation of reduced arabinotriose shows that two non-reducing α -1,5-linked arabinose units covering the subsites –1 and +1 are a prerequisite for hydrolysis.

3.1.2. Abn2

Abn2 degraded reduced arabinose oligomers with DP ≥ 3 , while enzyme activity increased with increasing DP of the substrate. From

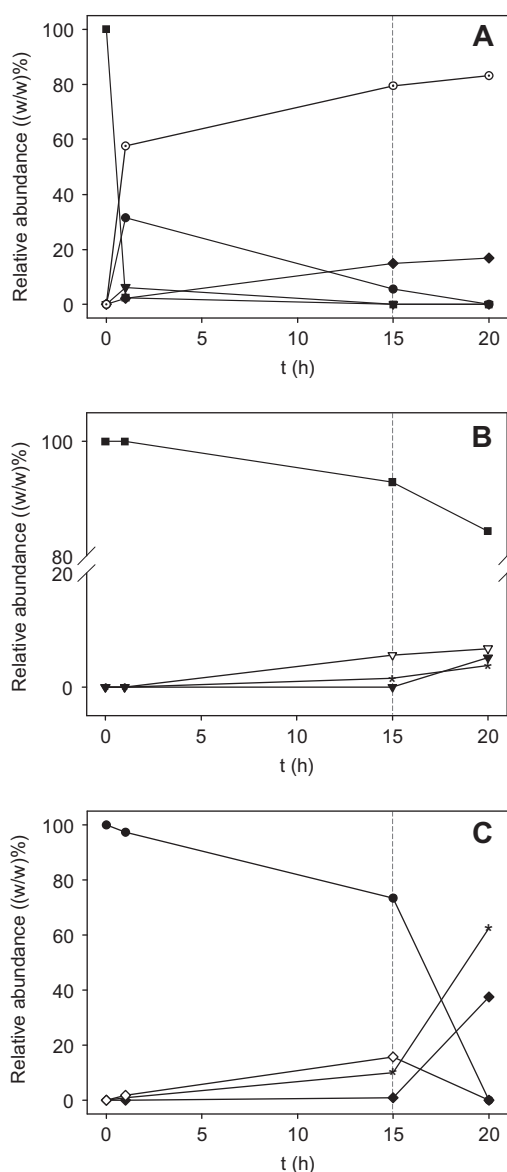


Fig. 2. Time curves of C1 arabinohydrolases Abn2, Abn4 and Abf3 towards pure, reduced arabinose oligomers. (A) Abn2 towards reduced arabinose heptaose. (B) Abn4 towards reduced arabinose heptaose. (C) Abf3 towards reduced arabinotriose. \blacklozenge – arabitol, \diamond – reduced arabinobiose, \bullet – reduced arabinotriose, \blacktriangledown – reduced arabinopentaose, \triangledown – reduced arabinohexaose, \blacksquare – reduced arabinose heptaose, $*$ – arabinose, \circ – arabinobiose, and grey line – fresh 10-fold enzyme dose added after 15 h incubation.

Fig. 2A it can be seen that Abn2 rapidly degraded reduced arabinose heptaose to reduced arabinopentaose and, further on, to arabinotriose by the subsequent removal of arabinobiose from the non-reducing end of the substrate. The data suggest that Abn2 binds the two arabinose units at its substrate binding subsites –1 and –2. The ability of Abn2 to degrade reduced arabinotriose implies that Abn2 can tolerate or does not recognize a reducing end or an alditol at the +1 subsite. Since Abn2 activity decreased with decreasing DP it is likely that Abn2 also binds parts of the substrate on one or more + subsites. Carapito and co-workers determined the crystal structure of a GH family 93 arabinobiohydrolase from *Fusarium graminearum* (2009). They describe the substrate binding site as a cleft with a very defined –1 subsite and a more open –2 subsite. Towards the reducing end, only the +1 subsite could be identified from where the cleft opens and does not allow any further prediction of additional subsites (Carapito et al., 2009). Abn2 also belongs to GH family 93 and has the same mode of action as the *F. graminearum* arabinobiohydrolase. An Abn2 substrate binding site that is structurally similar to the *F. graminearum* GH 93 arabinobiohydrolase substrate binding site would be in agreement with the experimental data obtained for Abn2.

3.1.3. Abn4

Abn4 is a GH family 43 arabinofuranosidase that is mainly active towards the side chains of branched arabinan. It can partly degrade linear arabinose oligomers with DP ≥ 4 in the given time (Kühnel et al., 2010). In contrast, Abn4 was not active towards reduced arabinotetraose and reduced arabinopentaose (no further data shown). Reduced arabinohexaose and reduced arabinose heptaose were partly degraded to yield reduced arabinopentaose and reduced arabinohexaose, respectively (Fig. 2B). These data show that Abn4 removes a terminal arabinose unit from the non-reducing end of the molecule. The alditol could decrease the substrate binding to Abn4 and, by this, reduce enzyme action. Alternatively, the alditol could not be recognized by Abn4 and, therefore, Abn4 action towards reduced arabinose oligomers is reduced.

In general, topologies of substrate binding sites are not highly conserved in GH family 43 enzymes (Nurizzo et al., 2002; Vandermarliere et al., 2009). However, these enzymes have a five-bladed β -propeller as a common feature (<http://www.cazy.org/GH43.html>). The binding site of endo enzymes has a cleft topology. In exo enzymes, like *G. stearothermophilus* GH 43 β -xylosidase and *Streptomyces avermitilis* exo-1,5- α -arabinofuranosidase, this cleft is blocked or closed on one side and forms a pocket like topology (Brüx et al., 2006; Fujimoto et al., in press). Low Abn4 activities towards linear arabinose oligomers and higher activities towards polymeric branched arabinan could suggest that Abn4 has a substrate binding site that recognizes a larger part of the substrate. The binding of the molecule would then occur with an endo-mode of action, whereas the catalytic activity itself would follow an exo-mode of action.

According to this data the arabinofuranosidase Abn4 is not part of the arabinofuranosidase family A or B or arabinoxylan arabinofuranohydrolase as described by Beldman et al. (1997). Structural characterization of Abn4 (e.g. by crystallography) could give more insight into the structure–function relationship.

3.1.4. Abf3

Abf3 is a GH family 51 arabinofuranosidase that releases arabinose from arabinoxylan and from linear and branched arabinose oligomers (Hinz et al., 2009; Kühnel et al., 2010). It can be seen from Fig. 2C that Abf3 removed single arabinose units from the non-reducing end of reduced arabinotriose. Abf3 activity towards reduced arabinose oligomers was 5–10 times lower than Abf3 activity towards pNP-Araf. It was most active towards reduced arabinobiose and the activity decreased with increasing DP of the

substrate. The clear preference for smaller oligomers and the lack of activity towards polymeric substrate indicates that Abf3 acts towards arabinose oligomers like expected for an arabinofuranosidase A (Beldman et al., 1997; Kühnel et al., 2010).

3.2. Product inhibition

Arabinobiose was added to Abn1 and Abn2 digests of linear arabinan since it is the main product that accumulates upon end point conversion. The reaction was followed by HPSEC (Fig. 3A and B). The linear arabinan polymer eluted at 9.8 min and was rapidly degraded to intermediate and small molecular mass materials that eluted from 11.5 to 15.0 min. The main peak at 14.75 min shows the increasing arabinobiose levels in the samples. The insert represents a zoom into the high molecular mass region in the range from 8.5 to 14.0 min.

3.2.1. Abn1

Abn1 was not inhibited in the degradation of linear arabinan even when 50 mg/ml arabinobiose were added to the mixture

(Fig. 3A). Some variation in the presence of small molecular mass material was observed. Digests with high concentrations of arabinobiose (20–50 mg/ml) contained more oligomers that eluted at 13.2, 13.5 and 13.9 min than digests with less arabinobiose. These changes are mainly considered to be derived from impurities within the arabinobiose preparation that was used in the experiment (as shown in Supplementary data, Fig. S3). When calculating the molar substrate to product ratios, it can be seen that even a 1700-fold product concentration did not lower Abn1 activity (Table 2). The data also correlates with the activities measured towards reduced arabinose oligomers (Fig. 1A), in which a decrease in enzyme efficiency was observed with a decrease of the degree of polymerization of the substrate. It suggests that the +2 subsite in Abn1 is not only important for Abn1 activity, but also for substrate binding.

3.2.2. Abn2

Unlike Abn1, Abn2 was very sensitive to product inhibition (Fig. 3B). The addition of 1 mg/ml arabinobiose reduced Abn2 activity to about 75% and the activity further declined to 20% and

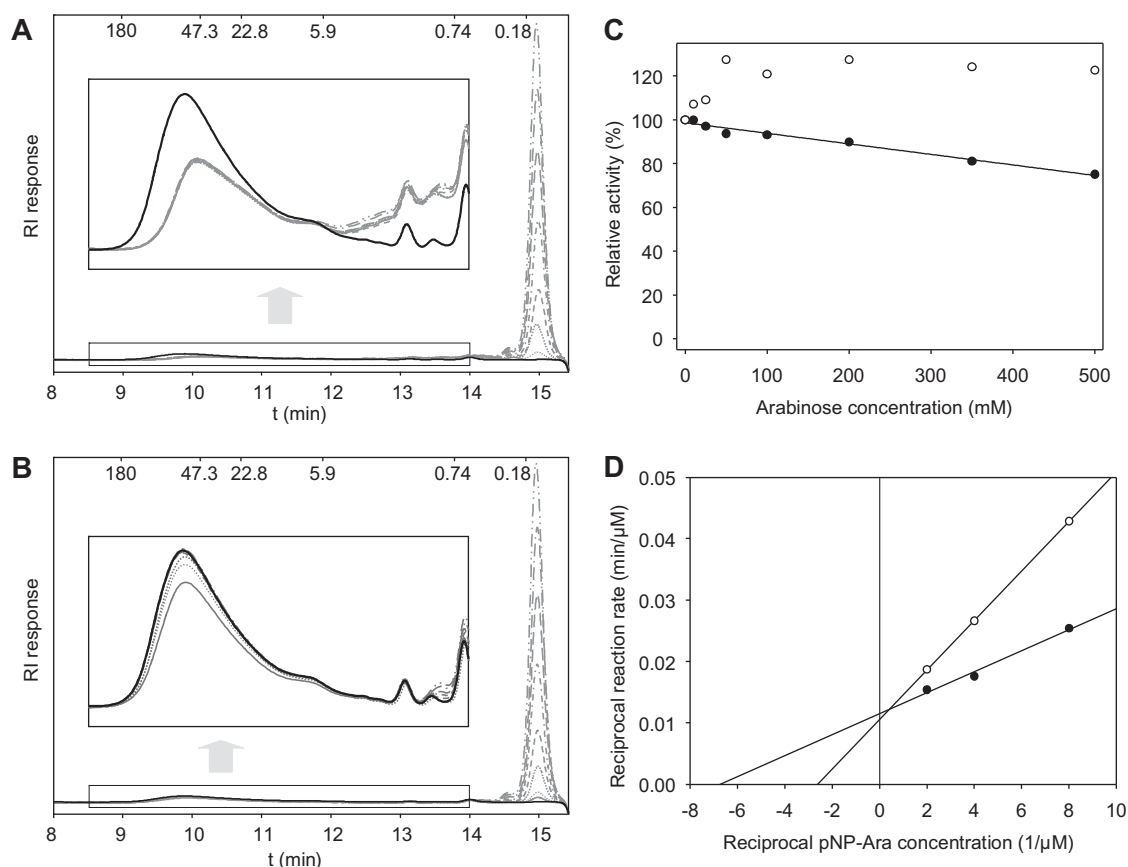


Fig. 3. Product inhibition of C1 arabinohydrolases. Linear arabinan degradation by Abn1 (A) and Abn2 (B) in the presence of 0–50 mg/ml arabinobiose ($t = 14.75$ min), upper X-axis: molecular masses of pullulan standards (in kDa), black line – blank, grey line – 0 mg/ml arabinobiose, grey dashed/dotted lines – 1, 5, 10, 20, 30, 40, 50 mg/ml arabinobiose with increasing peak area at 14.75 min. (C) Abn4 (○) and Abf3 (●) activity towards pNP-Araf (0.5 mM) in the presence of 0–500 mM arabinose. (D) Lineweaver-Burke-Plot of Abf3 towards pNP-Araf (from 0.125 to 0.500 mM) with 0 mM arabinose (●) and 500 mM arabinose (○).

Table 2

Product to substrate ratios for linear arabinan and arabinobiose concentrations used in the product inhibition experiment. A molecular mass of 50 kDa is assumed for linear arabinan and 282 Da for arabinobiose. Masses were used in the assay.

	Linear arabinan		Arabinobiose						
m (mg)	0.5	0.1	0.5	1	2	3	4	5	
n (μmol)	0.01	0.35	1.77	3.55	7.09	10.64	14.18	17.73	
Ratio	1	35	177	355	709	1064	1418	1773	

5% at concentrations of 5 and 10 mg/ml arabinobiose, respectively, as estimated from the surface area decrease in the range of 8.5–14.0 min. When calculating the molar ratios and assuming only one non-reducing end for a linear arabinan molecule, a 200-fold product concentration inhibited the Abn2 activity to 95%. Since linear arabinan is produced from branched arabinan, one molecule of linear arabinan may have several non-reducing ends that may be a substrate for Abn2. Therefore, the apparent ratio of inhibition could be even lower. Since the number of non-reducing ends in linear arabinan is not known, no inhibition constant was calculated.

Table 3

Branched arabinose oligomer structures according to Westphal et al. (2010a, in press). Horizontal white dots – α -1,5-linked arabinofuranose units, branches attached on the lower left side – α -1,3-linked arabinofuranose side chain, branches attached on the lower right side – α -1,2-linked arabinofuranose side chain.

Component structure	Schematic	PGC–HPLC–MS retention time (min)
1.0		<1.0
2.0		3.5
3.0		7.5
4.0		9.7
5.0		11.4
3.1		7.2
4.1		8.5
4.2		9.4
5.1		10.4
5.2		10.8
6.1 ^a		11.8
6.2		12.2
7.2 ^b		13.2
8.1		13.9

^a Predicted structure confirmed by the degradation of 8.1 by Abf3 and Abn1.

^b Proposed structure based on the degradation of 8.1 by Abn4.

Both enzymes were also incubated with linear arabinan in the presence of 150 mg/ml arabinose. None of the enzymes showed any product inhibition. Therefore, a specific binding of arabinose by Abn1 and Abn2 can be excluded (no further data shown).

3.2.3. Abn4 and Abf3

Exoarabinanase Abn4 did not show any product inhibition when incubated with arabinose (Fig. 3C). On the contrary, 50 mM arabinose seemed to stabilize the enzyme and lead to a 30% increase in enzyme activity. This increase in activity was also observed when glucose was added to the mixture. However, 50 mM sodium acetate buffer, xylose or inositol did not have any effect on the enzyme activity (no further data shown). It can be concluded that Abn4 is positively affected by the presence of certain sugars.

The Abf3 activity was reduced by 20% in the presence of 500 mM arabinose (Fig. 3C). A linear correlation between product concentration and Abf3 activity was observed. Abf3 digests with different initial substrate concentrations were analyzed to determine the inhibition mechanism. From the Lineweaver–Burke-Plot shown in Fig. 3D it can be seen that Abf3 product inhibition followed a competitive mechanism. The product can bind to the active site with a much lower affinity than the substrate. Abf3 activity is fully recovered when the substrate concentration is increased to 5 mM pNP-Araf.

3.3. Action of C1 arabinohydrolases towards branched arabinose oligomers

3.3.1. Action towards branched arabinopentaose isoforms

The activities of Abn4 and Abf3 towards branched arabinose oligomers were tested to determine the mode of action of the enzymes. The branched pentamers used consist of three α -1,5-linked arabinose units that carry two arabinose side groups. The middle

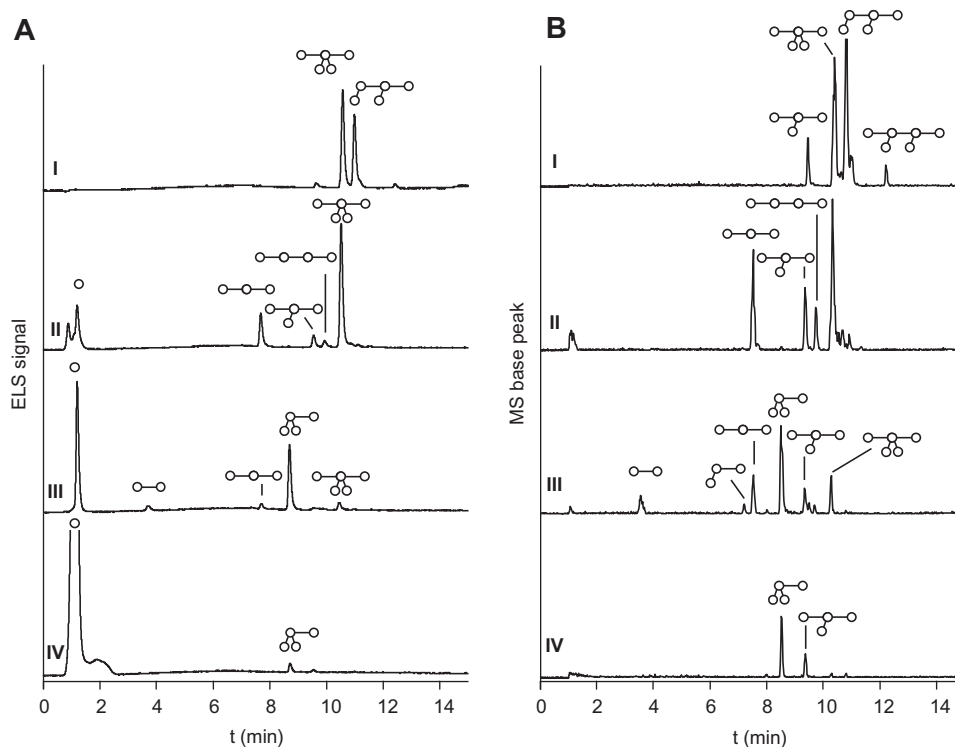


Fig. 4. Time dependent degradation of the arabinopentaose isomers by Abf3 and Abn4. (A) PGC–HPLC–ELSD retention time profiles of the branched arabinopentaose mix (BAP) and (B) PGC–HPLC–MS retention time profiles of BAP. (I) BAP (blank), (II) BAP digested with Abn4, (III) BAP digested with Abf3, and (IV) BAP digested with 10-fold concentration Abf3 ($t = 3$ h).

arabinose of the 5.1 isoform is double substituted with an α -1,2-linked and an α -1,3-linked arabinose, whereas the middle arabinose and the terminal non-reducing arabinose of the 5.2 isomer are single substituted with two α -1,3-linked arabinose units (Table 3). From Fig. 4A it can be seen that the branched arabinopentaose mix mainly contained these two structures that were differently degraded by Abn4 and Abf3 (lines II–IV). The evaporative light scattering (ELS) signal allows the quantification of the compounds present and the formation of the arabinose monomers. Fig. 4B shows the MS signal of the same samples in which minor compounds are more pronounced (e.g. the 4.2 and 6.2 oligomers, Fig. 4B, line I) than in the ELS signal.

Abn4 could only degrade the 5.2 isomer (Fig. 4, line II). It released the α -1,3-linked side groups from the pentamer 5.2 which lead to the release of arabinose and arabinotriose as the end products of the reaction. The oligomer 4.2 (Table 3) was formed as an intermediate product. This could suggest that the terminal α -1,3-linked arabinose at the end of the molecule is preferred over the α -1,3-linked arabinose attached to the second arabinose. Linear arabinotetraose was recovered from the Abn4 digestion mixture, which was derived from the branched hexamer 6.2 (Fig. 4, line II). The pentamer 5.1 could not be degraded by Abn4. It can, therefore, be concluded that Abn4 cannot hydrolyze the linkages of double substituted arabinose from arabinose oligomers.

In contrast to Abn4, Abf3 could degrade both branched arabinopentaose isomers over time (Fig. 4, line III). The amount of enzyme required for the degradation was similar to the amount needed for the degradation of the linear pentamer. The double substituted isomer 5.1 was converted into 4.1 ($t = 8.5$ min) by the removal of the terminal α -1,5-linked arabinose residue from the non-reducing end of the molecule. The 4.1 peak accumulated over time and was further degraded to monomer when a 10-fold overdose of Abf3 was added to the mixture (Fig. 4, line IV). The isomer 5.2 was degraded to the structure 4.2 by the hydrolysis of the terminal α -1,3-linkage at the non-reducing end (Fig. 4B, line III, $t = 9.4$ min). Subsequently, the molecule was further degraded to two trimers 3.0 and 3.1 that eluted at 7.5 and 7.2 min, respectively. Both trimers were then converted to arabinobiose and subsequently to arabinose as end product (Fig. 4, line IV). With respect to Abf3, it can be concluded that it can degrade all linkages present in 5.1 with a clear preference for single substituted sugars in proximity of the non-reducing end. The double substituted arabinose side chains are also degraded. However, the hydrolytic performance is 10-fold lower compared to the hydrolysis of a single α -1,3-linkage or α -1,5-linkage. The absence of any intermediate degradation products smaller than the 4.1 oligomer is in support of this behavior (Fig. 4A).

3.3.2. Action towards branched arabinooctaose

The C1 arabinohydrolases Abn1, Abn4 and Abf3 were sequentially tested towards the branched arabinooctaose isomer 8.1 to get more insight in structural limitations of enzymatic degradation. From Fig. 5A it can be seen that Abn1 was not active towards 8.1 (line II) whereas Abn4 had a low activity towards 8.1. It removed one arabinose unit and produced an arabinoheptaose isomer that eluted at 13.2 min (line III). This isomer was degraded by Abn1 to the known arabinopentaose isomer 5.1 and arabinobiose (line IV). Therefore, the structure of the arabinoheptaose released by Abn4 was concluded to be as shown in structure 7.2 (Table 3). Abn4 released the single substituted α -1,3-linked arabinose units from 5.2. It is likely that the neighboring double substituted arabinose residue sterically hindered the binding of the substrate by Abn4. Small amounts of a hexamer were released upon prolonged incubation with a 50 times overdose of Abn4 (Supplementary data, Fig. S4). Therefore, Abn4 could also

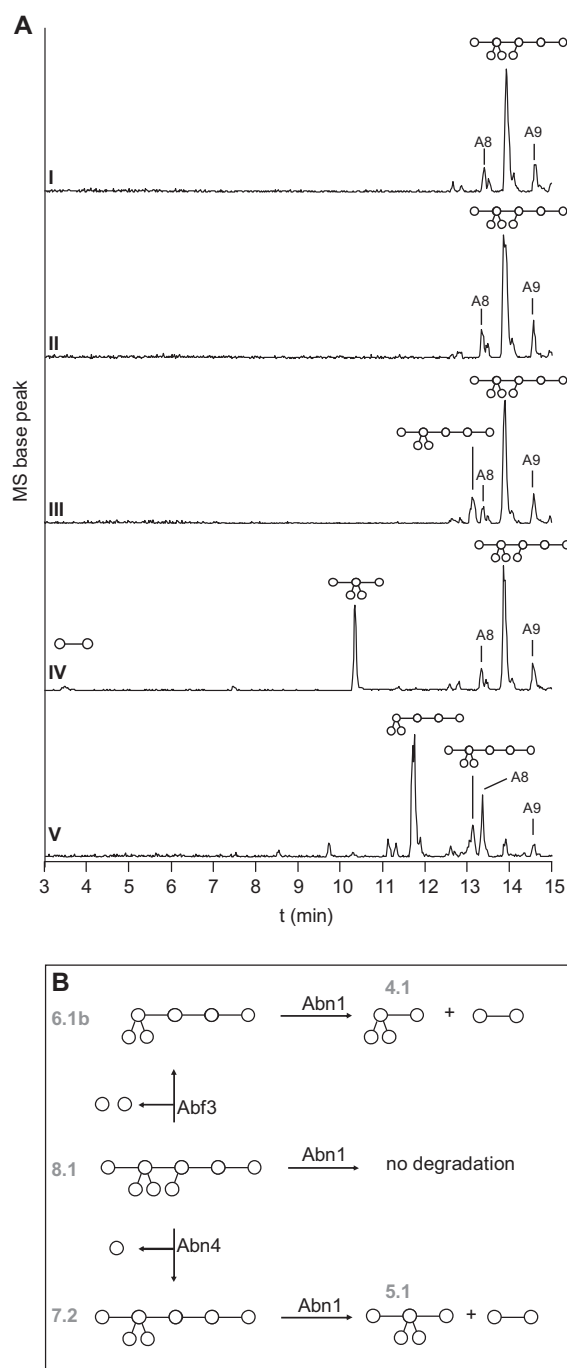


Fig. 5. Degradation of branched arabinooctaose 8.1 by C1 arabinohydrolases. (A) HPLC-MS elution profile of 8.1 (I), 8.1 treated with Abn1 (II), 8.1 treated with Abn4 (III), sample III treated with Abn1 (IV) and 8.1 treated with Abf3 (V). Structures are indicated when the component could be identified based on m/z ratio and retention time. Arabinose oligomers of unknown structure and known m/z ratio: A8 – arabinooctaose isomer, A9 – arabinononaose isomer. (B) Schematic representation of branched arabinooctaose degradation by Abf3, Abn4 and Abn1.

hydrolyze the α -1,5-linked arabinose at the non-reducing end. The speed of hydrolysis was very low, probably due to the presence of the neighboring double substituted arabinose unit.

No statement can be made regarding the performance towards α -1,2-linked arabinose, since no oligomers could be isolated that contain a single substituted α -1,2-linked arabinose (Westphal et al., 2010a). Nevertheless, it could be conjectured that Abn4 can also hydrolyze this linkage because sugar beet arabinan contains

4.5–8.0% α -1,2,5-linked arabinose (Beldman et al., 1993; Kaneko et al., 1998).

The data suggest that Abn1 is side chain tolerant. Abn1 can hydrolyze the α -1,5-linkage of the backbone despite the presence of a double substituted arabinose residue at subsite –2. The topology of the binding cleft of Abn1 allows it to host a double substitution. However, the hydrolytic performance is reduced when compared to the action towards linear arabinose oligomers.

Abf3 degraded 8.1 to the arabinohexaose isomer 6.1b (line V) that could be further degraded by Abn1 to give arabinobiose and 4.1 (no further data shown). It confirms that Abf3 can hydrolyze both, α -1,3-linked and α -1,5-linked arabinose. The appearance of the 7.2 isomer may imply a slight preference of the α -1,3-linked arabinose over the α -1,5-linked arabinose (line V) since this peak was the only arabinohexaose structure that was released by Abf3. Furthermore, Abf3 digest lead to an increase of the compound A8 eluting at 13.4 min. A8 is an arabinooctaose isomer of unknown structure. The early elution time suggests a highly branched structure that could be produced by the degradation of the arabinononaose isomer (A9) that eluted at 14.6 min.

4. Conclusions

The C1 arabinohydrolases have different modes of action and substrate specificities. The exoarabinanase Abn2 releases arabinobiose from the non-reducing end of the α -1,5-linked arabinan backbone and prefers, like Abn1, polymers over oligomers. Endoarabinanase Abn1 can hydrolyze substrates that carry a double substitution at the subsite –2 and is therefore considered as side chain tolerant. The arabinohydrolases Abn4 and Abf3 release arabinose monomers from the non-reducing end of the molecule with different modes of action towards natural substrates. The characterized enzymes allow the controlled and efficient degradation of arabinan to either monomers, or, by partial degradation, to branched arabinose oligomers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.biortech.2010.09.029](https://doi.org/10.1016/j.biortech.2010.09.029).

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